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HYPERVIRULENT KLEBSIELLA PNEUMONIAE BLOODSTREAM ISOLATES IN THE UNITED ARAB EMIRATES

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HYPERVIRULENT *KLEBSIELLA PNEUMONIAE* BLOODSTREAM ISOLATES IN THE UNITED ARAB EMIRATES

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Microbiology and Immunology)

Under the Supervision of Professor Tibor Pál

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Declaration of Original Work

I, Shaikha Awad Mohammed Saeed Shemail Al Kaabi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled *"Hypervirulent Klebsiella Pneumoniae Bloodstream Isolates in the United Arab Emirates"*, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Tibor Pál, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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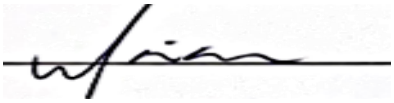
   Signature  Date 11-12-2019

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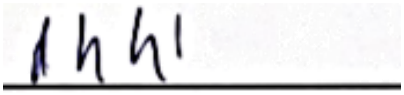
   Signature  Date 11-12-2019

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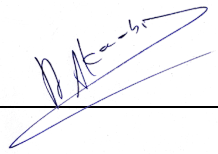
Signature  Date 13 Sept 2020

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Abstract

As the presence of hypervirulent *Klebsiella pneumoniae* (hvKP) has not been studied in the United Arab Emirates (UAE), the aim was to determine its rate among bloodstream isolates collected in a tertiary care hospital during a two-year period. *K. pneumoniae* blood isolates of 125 individual patients from Tawam Hospital, Al Ain in 2014-2015 were investigated. Clinical data on patients' demographics and underlying medical conditions were collected. Speciation was carried out by VITEK 2, antimicrobial susceptibility was determined by disc diffusion and for colistin by microdilution. Hypermucoviscosity was assessed by string test. Genetic markers of hypervirulence (*iucA, rmpA, rmpA2*), K1 and K2 serotype-specific alleles, and carbapenemase genes were detected by PCR. HvKP isolates were compared by PFGE and by MLST and the localisation of *rmpA* was confirmed by hybridization. Fischer's exact test was used to calculate correlations between categorical variables. Of the 125 isolates 13 (10.4%) *iucA* and *rmpA/rmpA2* positive strains were identified and were considered hvKP. Eight of them were string test positive. They were distributed among CC23 - serotype K1 (6, PFGE similarity >80%), ST65 – K2 (1), ST380 – K2 (2), ST86 – K2 (1), ST36 (1) and ST714 (2), respectively. While the majority of them were susceptible to most antibiotics tested, one *K. pneumoniae* ST23 was multi-drug resistant and produced a new allele of VIM carbapenemase. hvKP strains were more likely to be isolated from diabetic than from non-diabetic patients (P=0.001) and from patients who have not received prior antimicrobial therapy (P=0.0208). A further 11 *iucA* positive isolates were *rmpA/rmpA2* and string test negative. Ten of these strains produced OXA-232 carbapenemase and were extremely drug resistant (XDR). These isolates belonged to *K. pneumoniae* ST231. The results surmise that while members of this latter, carbapenem resistant group may not fully qualify as hvKP, yet, they carry the potential to develop into hvKP by acquiring the plasmid-coded exopolysaccharide synthesis regulator, *rmpA*. An alarming rate (>10%) of hvKP was demonstrated among bloodstream isolates studied with the presence of a carbapenem resistant, hyperviscous phenotype hvKP ST23 isolate producing a novel VIM-carbapenemase.

Keywords: Hypervirulent *Klebsiella pneumoniae* (hvKP), Hypermucoviscosity, String test, Virulence factors, Carbapenemase genes, Enterobacteriaceae.

Title and Abstract (in Arabic)

دراسة سلالات الكلبسية الرئوية الضارية المعزولة من مجرى الدم في الامارات العربية المتحدة

الملخص

نظرًا لعدم وجود دراسات كافية لسلالات بكتيريا الكلبسية الرئوية الضارية (hvKP) شديدة الضراوة في دولة الإمارات العربية المتحدة، كان الهدف هو تحديد معدلاته بين العينات المعزولة من مجرى الدم التي تم جمعها في مستشفى قطاع الصحة الثالث خلال فترة عامين.

تم فحص ما يعادل 125 عينة من بكتيريا الالتهاب الرئوي المعزولة من 125 مريضًا في مستشفى توام، العين في الفترة ما بين 2014-2015. تم جمع البيانات السريرية والحالات الطبية الأساسية للمرضى. تم تعريف البكتيريا عن طريق ال VITEK 2، وتم تحديد القابلية للمضادات الحيوية عن طريق ال disc diffusion، اما بالنسبة لمضاد الكوليستين فتم اختباره عن طريق التخفيف الجزئي. تم تقييم فرط المخاطية عن طريق اختبارال (String). تم الكشف عن علامات جينية لتحديد مدى ضراوة العينات (iucA, rmpA, rmpA2)، تم تعيين ال K1 و K2 الخاصة بالنمط المصلي، وكذلك جينات ال carbapenemases بواسطة ال PCR. تمت مقارنة عينات ال hvKP بواسطة تقنية ال PFGE و MLST، تم تحديد وجود جين ال rmpA بواسطة ال hybridization. تم استخدام اختبار فيشر الدقيق لحساب الارتباطات بين المتغيرات الفئوية للفئات المختلفه.

بناء على دراسة ال 125 عينة، تم ايجاد 13 (10.4%) عينة موجبة لجينات (iucA, rmpA/rmpA2) واعتبرت عينات شديدة الضراوة (hvKP). ثمانية منهم كانت ايجابية لاختبار ال (string) وتم توزيعها بين الانماط التالية CC23 - K1 (6), ST65 - K2 (1), ST380 - K2 (2), ST86 - K2 (1), ST36 (1) ST714 (2) على التوالي. على الرغم من أن معظم عينات ال (hvKP) كانوا شديدي الحساسية لمعظم المضادات الحيوية التي تم اختبارها، إلا أن أحد أنواع هذه البكتيريا المنتمية لنمط ال ST23 كان مقاومًا لعدة مضادات وأنتج نوعا جديدًا من جين ال (VIM). تم عزل سلالات ال hvKP أكثر من مرض السكري مقارنةً بالمرضى غير المصابين بالسكري (P=0.001) وكذلك من المرضى الذين لم يتلقوا اي علاج سابق بالمضادات الحيوية (P=0.0208).

كذلك بينت الدراسات ان هناك 11 عينة موجبة لجين ال iucA ولكنها سالبة لكل من جينات ال (rmpA/rmpA2) وكذلك لاختبارال (string). أنتجت عشر من هذه السلالات جين ال OXA-232وكانت مقاومة للغاية للأدوية (XDR). تنتمي هذه العينات إلى الكلبسيه الرئوية من النمط ST231، ونحن نفترض أنه على الرغم من أن أعضاء هذه المجموعة الأخيرة، مجموعة ال OXA-232 المقاومة للكاربابينم قد لا ينتمون الى السلالة الضارية hvKP شديدة الضراوة، إلا أنهم يحملون القدرة على التطور إلى هذه السلالة من خلال الحصول على جين ال rmpA المنقول بواسطة البلازميدات.

تم إثبات وجود معدل ينذر بالخطر (10%<) من السلالات الضارية الشديدة الضراوه (hvKP) بين العينات المعزولة من مجرى الدم التي تمت دراستها مع وجود عينة للنمط ST23 المقاوم للكاربابينيم، والمنتج لنوع جديد من جين ال VIM.

مفاهيم البحث الرئيسية: بكتيريا الكلبسية الرئوية الضارية (hvKP)، فرط المخاطية، اختبارال (String)، مضادات الحيوية، علامات جينية، جينات ال Carbapenemases، كاربابينيم.

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Dedication

To my Country UAE, Community in Al Ain City, to UAEU and to Tawam Hospital and to my beloved parents, family and friends

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List of Abbreviations

°C	Degree Celsius
A. baumannii	*Acinetobacter baumannii*
AK	Amikacin
AP	Ampicillin
API	Analytical Profile Index
ATCC	American Type Culture Collection
AUG	Augmentin (Amoxicillin and Clavulanic Acid)
AZT	Aztreonam
BA	Blood Agar
BC	Blood Culture
BLNAR	ß-lactamase Negative, Ampicillin Resistant
ß-lactam	Beta-lactam
ß-lactamase	Beta-lactamase
bp	Base Pairs
BSI	Bloodstream Infection
CAZ	Ceftazidime
CC	Clonal Complex
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CG	Clonal Group
CHL	Chloramphenicol
CIP	Ciprofloxacin
cKP	‘classic’ *K. pneumoniae*
CLSI	Clinical and Laboratory Standards Institute
CMS	Colistin Methanesulfonate (Colistimethate)
CNS	Central Nervous System
COL	Colistin
cont.	Continued
CPD	Cefpodoxime
CRE	Carbapenem Resistant Enterobacteriaceae
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*

CSKP	Carbapenem Susceptible *Klebsiella pneumoniae*
CPS	Capsular Polysaccharide
CTX	Cefotaxime
CTX-M	Cefotaximase
CXM	Cefuroxime
DBO	Diazabicyclooctane
DOS	Disubstituted 2-deoxystreptamine
DNA	Deoxyribonucleic Acid
dNTP's	Deoxynucleoside Triphosphates
DPA	Dipicolinic Acid
DXT	Doxycycline
E. aerogenes	*Enterobacter aerogenes*
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetraacetic Acid
ERT	Ertapenem
ESBL	Extended Spectrum ß-Lactamase
Fe^{3+}	Ferric ion Iron (3^{+})
FOS	Fosfomycin
FOX	Cefoxitin
GN	Gentamicin
GI	Gastrointestinal Tract
HGT	Horizontal Gene Transfer
HMKP	Hypermucoviscous *Klebsiella pneumoniae*
hvKP	Hypervirulent *Klebsiella pneumoniae*
IMP	Imipenem
IS	Insertion Sequence
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* Carbapenemase
LPS	Lipopolysaccharide
magA	Mucoviscosity Associated Gene
MBL	Metallo ß-lactamases
mcr	Mobile Colistin Resistance

MDR	Multi-drug Resistance
MDRO	Multi-drug Resistance Organism
MEM	Meropenem
M. morganii	*Morganella morganii*
mg	Milligram
MHA	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
min	Minute
mL	Millilitre
MLST	Multi-locus Sequence Typing
mM	Millimolar
mm	Millimetre
MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Not Applicable
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NDM	New-Delhi Metallo-beta-lactamase
ng	Nanogram
NICU	Neonatal Intensive Care Unit
nm	Nanometre
OM	Oman
OMP	Outer Membrane Protein
OS	Oligosaccharide
OXA	Oxacillinase
P. aeruginosa	*Pseudomonas aeruginosa*
PBPs	Penicillin Binding Proteins
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
PEtN	Phosphoethanolamine
PFGE	Pulsed Field Gel Electrophoresis
PLA	Pyogenic Liver Abscess
P. mirabilis	*Proteus mirabilis*

PMQR	Plasmid-mediated Quinolone Resistance
P. polymyxa	*Paenibacillus polymyxa*
pH	Potential Hydrogen, measure of acidity or basicity of a solution
PTZ	Piperacillin-tazobactam
rRNA	Ribosomal RNA
rmpA	Regulator of the Mucoid Phenotype
RMTs	Methyltransferases
RND	Resistance Nodulation Cell Division
rpm	Revolutions Per Minute
RT	Room Temperature
s	Seconds
S. aureus	*Staphylococcus aureus*
S. maltophilia	*Stenotrophomonas maltophilia*
S. marcescens	*Serratia marcescens*
SDS	Sodium Dodecyl Sulfate
SHV	Sulfhydryl Reagent Variable Enzyme
spp.	Species
SLV	Single Locus Variant
ST	Sequence Type
SXT	Trimethoprim-Sulphamethoxazole
TBE	Tris/Borate/EDTA
TEM	Temoneira
TE	Tris/EDTA Buffer
TGC	Tigecycline
TOB	Tobramycin
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
tRNA	Transfer Ribonucleic Acid
UAE	United Arab Emirates
UK	United Kingdom
UTI	Urinary Tract Infection
VIM	Verona Integron-encoded Metallo-β-lactamase
VRE	Vancomycin-Resistant *Enterococcus*

VRSA	Vancomycin-Resistant *Staphylococcus aureus*
WHO	World Health Organization
µg	Micro-gram
µl	Micro-litre
µM	Micro-molar

Chapter 1: Introduction

1.1 Enterobacteriaceae

The *Enterobacteriaceae* family is the largest heterogeneous group of medically important Gram-negative bacteria. Currently it contains 50 genera and dozens of named species as well as subspecies. Initially, they were classified according to their biochemical properties, antigenic structure and subsequently by DNA-DNA hybridization and 16S rRNA gene sequencing. Most members of the family are ubiquitous organisms besides their human and animal hosts as part of the normal flora, are also found in soil and water (Murray, 2013).

They are Gram-negative, non-spore straight rods relatively small in size (0.3 to 0.3 to 1.0 x 1.0 to 6.0 μm). Some genera are motile by means of peritrichous flagella, meanwhile others are non-motile (Murray, 2013).

Members of *Enterobacteriaceae* family exhibit varying levels of pathogenicity. Those that are members of the normal flora can cause infections only if displaced to other body-sites (e.g. *Escherichia coli* from the gut to the urinary tract). Others are strict pathogens, like members of the *Salmonella* genus, while several of them cause mainly opportunistic infections, i.e. exclusively in patients with compromised defense systems (e.g. *Serratia marcescens*). The subject of the thesis is an important member of this family belonging to the genus *Klebsiella,* i.e. *K. pneumoniae* (Murray, 2013).

1.2 The genus Klebsiella and Klebsiella pneumoniae

The intensive recent research directed towards the genus *Klebsiella* has resulted in considerable fluidity in its taxonomy that is far from a complete consensus. According to the Taxonomy Browser of the NCBI the genus contains several species and subspecies of which the most important ones are *K. pneumoniae, K. oxytoca, K. rhinoscleromatis, K. quasipneumoniae,* and *K. variicola* (Wyres and Holt, 2016). *K. pneumoniae* are fermentive bacteria which produce lysine decarboxylase but not ornithine decarboxylase and are generally positive in the Voges-Proskauer test (Enjalbert *et al.*, 1979). They are non-motile, facultative anaerobes and it can grow rapidly on variety of selective and non-selective media. The colony appearance on the media is typically large, mucoid (wet, heaped, viscous colonies) due to a prominent capsule (Murray, 2013; Podschun and Ullmann, 1998).

It was first described by the German microbiologist Edwin Klebs (1834 – 1913) in 1875 while examining the airways of patients who died due to pneumonia. Eventually, the species was formally described by Carl Friedlander in 1882 and the organism was named "*Klebsiella pneumoniae*" by Trevisan in 1885 (Etymologia: Klebsiella *et al.*, 2010) (Friedländer, 1882). To honor *Friedlander's* contribution, the multilobar bronchopneumonia often leading to pulmonary abscesses and lung necrosis, typically seen in cases caused by *K. pneumoniae* used to be called "*Friedlander pneumonia".* Currently, *K. pneumonia* is one of the important causes of human morbidity and mortality (Wyres and Holt, 2016).

1.3 The pathogenicity of *Klebsiella pneumoniae*

K. pneumoniae is responsible for numerous infections within hospitals, long term care facilities as well as in the communities worldwide and (Carpenter, 1990). It is often described as an opportunistic pathogen as, nowadays, most infections are seen in hospitals among inpatients with varying degrees of immunocompromisation. It should be kept in mind, however, that although it is part of the normal flora, under certain circumstances, they have the ability to cause severe illnesses, such as pneumoniae or bacteremia, meningitis and other infections in non-immunocompromised people, as well (Figure 1) (Murray, 2013).

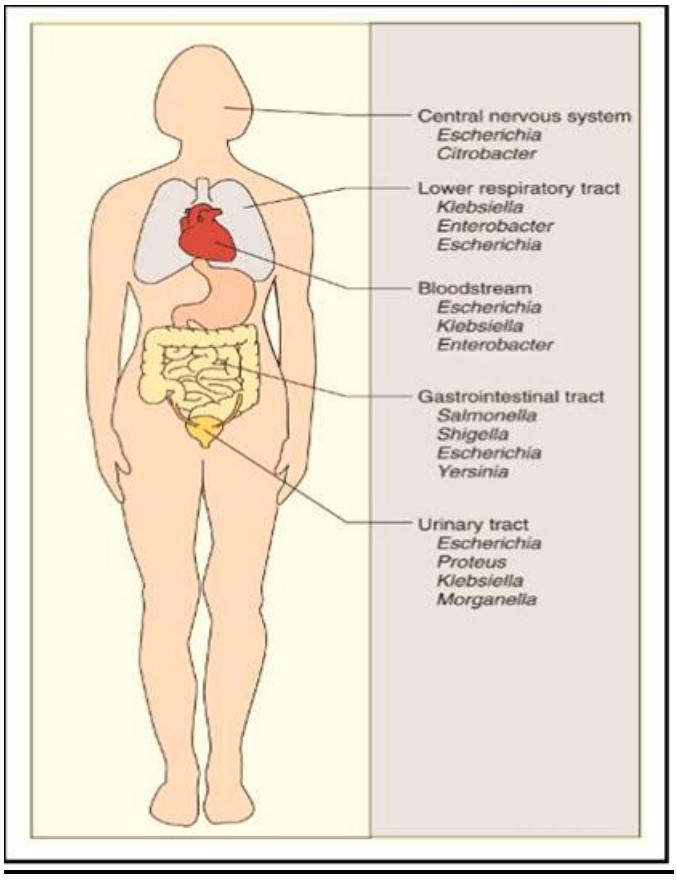

Figure 1: Sites of infections (Murray, 2013)

Klebsiella spp. are ubiquitous in nature, in environments, such as in water, sewage, soil and plants (Morrow *et al.*, 1978; Wyres and Holt, 2018). In addition to the hospital settings and medical devices, in association with the human host, *K. pneumoniae* is usually found in the mouth, skin and intestines. Generally, the colonization of the GI tract by *K. pneumoniae* takes place before the development of nosocomial infections (Podschun and Ullmann, 1998). It has been found that *K. pneumoniae* biofilms that appear on medical devices (e.g., catheters and endotracheal tubes) plays a significant role in infection of catheterized patients (Schroll *et al.*, 2010). Studies by Jagnow and Clegg (2003) showed that nosocomial infections caused by *K. pneumoniae* have the tendency to be persistent due to the presence of *K. pneumoniae* biofilms in-vivo which protects the bacteria from attacks of the host immune responses and from antibiotics (Jagnow and Clegg, 2003). In addition, nosocomial isolates of *K. pneumoniae* often exhibit multidrug-resistance (Paterson *et al.*, 2004; Munoz-Price *et al.*, 2013). Such resistance factors considerably increase the ability of these organisms to spread widely and rapidly which will often lead to nosocomial outbreaks within the hospital, making it one of the most common organisms causing such epidemics (Kuhn *et al.*, 1993).

Taken together, *K. pneumoniae* is a globally encountered pathogen that remains a significant cause of both community-acquired and healthcare-associated infections, including bacteremia. Currently, with the increase of hospitalized and immunocompromised patients, most infections are encountered nosocomially among individuals suffering from various underlying conditions (Shon *et al.*, 2013).

There are two major factors that make *K. pneumoniae* one of the most important pathogens of recent times:

- its broad pathogenic potential and
- its proclivity to become resistant to antibiotics.

1.3.1 Virulence factors

The disease-causing capacity of an organism, in relation to a specific host, is called “pathogenicity”, while, its quantitative expression is called “virulence”. Both terms refer to the capacity of a microorganism to replicate and disseminate and cause disease or kill the host. Virulence factors are molecules and cells structure components with functions essential in the pathogen–host interaction during the process of infection. These traits enhance the ability of bacteria to cause pathological changes within the host (Baer and Harold, 1956; Murray, 2013; Clegg *et al.*, 1992; Rosen *et al.*, 2008).

K. pneumoniae exhibit complex interactions with the host, and hence, a number of bacterial factors have been identified that contribute to the pathogenesis of these infections Figure 2 and Table 1.

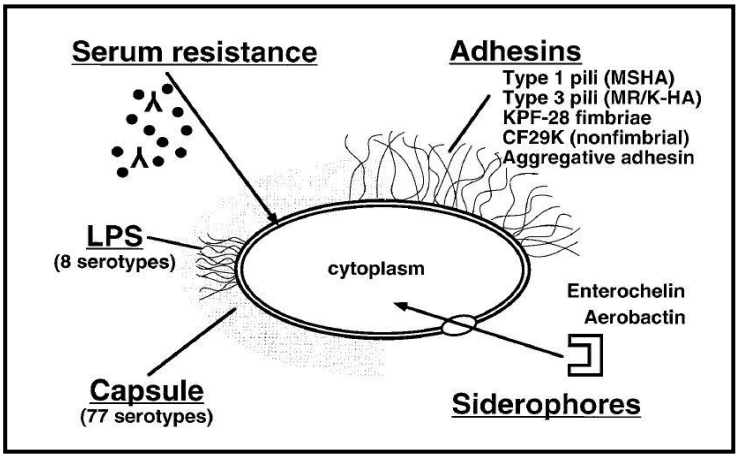

Figure 2: Pathogenicity factors of Klebsiella (Ottow, 1975)

Table 1: Virulence factors involved in the pathogenesis of *Klebsiella pneumoniae* (Clegg and Murphy, 2016)

Factor	Function
Capsule	Inhibit and evade phagocytosis by host cells, induces dendritic cell maturation, neutralizes antibacterial activity of host defense
LPS	O antigen provides serum resistance
Siderophore	Scavenge essential iron for survival, hypermucoviscous phenotypes have been linked to increased iron-binding activity
Urease	Limited role in precipitation of inorganic salts leading to catheter encrustation
Type 1 fimbriae	Involved in the formation of intracellular bacterial communities
Type 3 fimbriae	Important for biofilm formation on biotic and abiotic surfaces, role in biofilm formation on urinary catheters in vivo remains to be elucidated
Biofilm formation	Formation promotes resistance to host killing and antimicrobials, experimentally shown to be facilitated in part by fimbriae and capsule
Antibiotic resistance	Carbapenem-resistance prevents many treatment options

1.3.1.1 Capsular antigens

Two major components are expressed on the surface that are essential for the virulence of *K. pneumoniae:* the capsular polysaccharide (CPS; the K antigen) and the lipopolysaccharide (LPS; the O antigen) (Follador *et al.*, 2016) *K. pneumoniae* contains a thick capsule layer surrounding the bacterium (160 nm) that is generally considered an important factor in its pathogenicity (Figure 3).

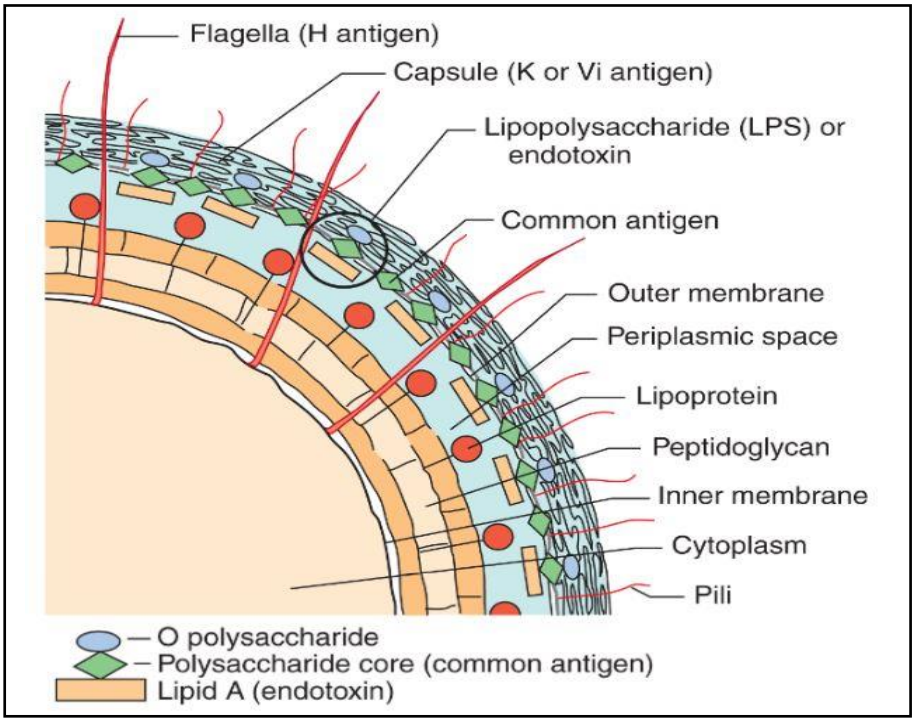

Figure 3: Cell wall structure of Enterobacteriaceae (Murray, 2013)

For a bacterium to cause infections, the first step is to enter and colonize the host and in this the capsule plays important roles. The capsular material forms a thick bundle of fibrillous structures that covers the bacterial surface. The capsule is strongly antiphagocytic, protecting the bacteria from phagocytosis and from bactericidal serum factors. Furthermore, it can act as a barrier to toxic hydrophobic molecules, like detergents (Murray, 2013).

Strains of *K. pneumoniae* can express several antigenically distinct capsules and some are considered to be associated with increased virulence potential. The capsule antigens K1, K2, K4 and K5 are mainly associated with high virulence degree (Kato, 1983). The degree of virulence conferred by a particular K antigen might be connected to the expression of type 1 fimbriae (a mannose receptor-specific adhesin) (Podschun and Ullmann, 1992; Ohman, 1993).

The genes responsible for the production of capsules by *K. pneumoniae* were originally designated as *cps* gene cluster and were mapped close to *his* on the bacterial chromosome (Clegg and Murphy, 2016). It has been discovered by cloning of the *cps* cluster that the genes are approximately 15kb of DNA and has all the determinants which are necessary to define the serotype of a strain (Clegg and Murphy, 2016). The studies showed that three alleles of a different gene named *rmpA/A2* have been described to play a role in the activation of *cps*-gene expression (Cheng *et al.*, 2010; Lai *et al.*, 2003).

The alleles vary between serotypes and the geographic region of the isolates. Two of the alleles are plasmid encoded and the third one is chromosomal. Over production of *rmpA* leads to a hypermucous phenotype in *K. pneumoniae* and to the increase in expression of the transcriptional fusions of *cps*-promoter regions (Lai *et al.*, 2003). The transcription of *rmpA* is inhibited by not only the *Fur* protein but also by other genes associated with iron-acquisition systems in *K. pneumoniae* (Lin *et al.*, 2011). For that reason, the concentration of available iron in the environment of the *Klebsiella* strains can play a major role in the expression of the virulence gene, including the amount of capsule produced by clinical isolates. The studies showed that K1 serotypes of *K. pneumoniae* are associated with causing severe disseminated infections. The mucoviscosity-associated gene (*magA*) was found to be part of the *cps*-gene cluster in these isolates and necessary for K1 biosynthesis (Fang *et al.*, 2010). Earlier, this gene has been used to identify, by polymerase chain reaction (PCR), K1-positive strains, but recently a similar allele was found in other K-serotypes (Fang *et al.*, 2010).

The *wzy* and *wzi* genes are present in all K types, but each one of them has a high level of variable sequence between distinct K types. Based on that, *wzy*-targeting PCR assays have been set to identify K1, K2, K3, K5, K20, K54 and K57 (Turton *et al.*, 2010; Fevre *et al.*, 2011), and by *wzi* sequencing one can reveal the K types of most clinical *K. pneumoniae* isolates (Brisse *et al.*, 2013).

Generally, the most virulent strains of *K. pneumoniae* produce a capsule made of saccharides that do not facilitate binding to phagocytic cells and hence are more resistant to phagocytosis (Clegg and Murphy, 2016).

1.3.1.2 Lipopolysaccharide (LPS)

Similarly to other members of the family *Enterobacteriaceae*, the lipopolysaccharides (LPSs) of *K. pneumoniae* consist of three structural domains shown on Figure 3, (i) the hydrophobic lipid A, which is a major component of the outer leaflet of the Gram-negative outer membrane and is responsible for the endotoxin activity and is an important virulence factor, (ii) the core oligosaccharide, which is linked to lipid A and provides the attachment site for (iii) the long chain polysaccharide (*O* antigen; *O* chain) (Follador *et al.*, 2016; Murray, 2013).

Beyond the endotoxic nature of LPS, the O antigen also contributes significantly to the virulence of *K. pneumoniae.* The presence of cell wall receptors enables *K. pneumoniae* to attach to the host cell. Correspondingly, LPS have been implicated in the pathogenesis of *K. pneumoniae* causing community-acquired pyogenic liver abscess (PLA) (Tomas *et al.*, 1997).

It has been shown in different studies that *K. pneumoniae* O-antigen prevents the access of complement components to activators (e.g., porins and rough LPS) and hence

contributes to bacterial resistance against complement-mediated killing (Merino *et al.*, 2000).

1.3.1.3 Pili (fimbriae) and adhesion

Pili or fimbriae are hairlike structures, 10 μm long and have a diameter of 1 to 11 nm (Jones and Isaacson, 1983). They are composed of polymeric globular protein subunits (pilin) and are protruding from the surface of the bacteria (Murray, 2013). As an adherence factor, fimbriae are important determinant of colonization.

The three *K. pneumoniae* fimbrial adhesins that have been described in detail are types 1 and 3 fimbriae and the Kpc fimbriae (Clegg *et al.*, 2011; Wu *et al.*, 2010). Type 1 fimbriae, also known as mannose-sensitive fimbriae, because of their capability to bind soluble mannose as a competitive inhibitor to binding, are usually encoded by the *fim* gene cluster. Type 3 fimbriae are encoded by the *mrk*-gene cluster and can be present in *K. pneumoniae* on a plasmid and/or a chromosomal gene cluster (Clegg and Murphy, 2016). It has been noted that *mrkA* is the major structural component of the fimbrial shaft and that *mrkD* helps to facilitate binding to extracellular- matrix proteins and act as the fimbrial adhesion (Clegg and Murphy, 2016).

The studies showed that the Kpc fimbriae are most frequently associated with K1-positive strains of *K. pneumoniae* causing disseminated pyogenic infections (Wu *et al.*, 2010). The production of these fimbriae is mediated by the *kpcABCD* gene cluster, although the conditions for optimal expression of these genes have not been yet fully understood.

The other type of *Klebsiella spp.* adhesin is the plasmid encoded CF29K. This fimbrial type is characterized by aggregative adhesion to the intestinal cell lines (Joly, 1992).

1.3.1.4 Serum resistance

The bactericidal effect of serum is one of the first line of defense by the host against invading microorganism. Serum resistance represented by activation of complement proteins. Complement components are activated in a cascade manner through 3 different pathways of activation namely classical, alternative or by the lectin pathway. The complement system plays a vital role in humoral defense against microbial pathogens. The serum bactericidal system has a very important role which is to prevent microorganisms from invading and persisting in the blood (Ramm *et al.*, 1983; Taylor, 1983; Old and Adegbola, 1985; Doorduijn *et al.*, 2016). In a study of Gharrah et al., 2017 serum resistance was one of the pathogenicity factors of *Klebsiella* and serum resistance was an indication of the higher pathogenicity (Gharrah *et al.*, 2017). Interestingly, studies found relationship between ESBL production and serum resistance showing that serum resistance was elevated in *K. pneumoniae* ESBL producer than non-ESBL producing *K. pneumoniae* (Lin *et al.*, 2016).

1.3.1.5 Iron acquisition

Iron is an essential factor in bacterial growth but the availability of free ferric iron (Fe^{3+}) in the host is usually extremely low. Consequently, pathogens, such as *K. pneumoniae* should be equipped to secure their iron supply from the host. This is achieved by a variety of mechanisms, most commonly either through ABC transporters and/or by secreting low-molecular-weight, high-affinity iron chelators, called

siderophores (Bullen *et al.*, 1978; Khimji and Miles, 1978; Griffiths, 1988; Behnsen and Raffatellu. 2016; Ellermann and Arthur, 2017)**.** Although the presence of siderophores are likely to be essential in all systemic infections, their role was mostly studied in a set of *K. pneumoniae* strains, called hypervirulent *K. pneumoniae.*

Approximately a dozen different systems have been identified that play a role in supplying iron to *K. pneumoniae* (Liu *et al.*, 2014) of which the siderophore-based systems are the most important (Table 2).

Table 2: The most common iron acquisition systems of K. pneumoniae (simplified from Liu et al., 2018)

System	Name	Genes
Kfu	ABC transporter	*kfuABC*
Fep-Ent	Enterochelin	*fepA-entD*, *fes-entF*, *fepDGC*, *ybdA*, *febB*, *entCEBA*
IroA	Salmochelin	*iroN*, *iroBCDN*
Iuc	Aerobactin	*iucABCD-iutA*
High-pathogenicity island	Yersiniabactin	*ybtPQXS*, *YbtA-irp2-irp1-ybtUTE-fyuA*

In clinical isolates these siderophore genes can be present in different combinations. Mutants lacking them are attenuated (Clegg and Murphy, 2016). A study of more than thirty strains of 23 different serotypes of *K. pneumoniae* showed that the siderophore enterochelin was the predominant iron-salvaging-system compound produced in urinary tract infections (Tarkkanen *et al.*, 1992). This was in contrast to bacteremic strains, mainly K1 and K2 serotypes, in which aerobactin was correlated to virulence. Some, particularly the aerobactin system is highly characteristic to strains with increased virulence (see later).

1.3.2 Hypervirulent (Hypermucoviscous) *Klebsiella pneumoniae*

Lately, the majority of infections caused by 'classic' *K. pneumoniae* (cKP) occurred in hospitals and long-term care facilities. Recently, however, a new type of infection emerged caused by more or a less distinct group of strains, initially called hypermucovicous, lately named hypervirulent *K. pneumoniae* (hvKP). Often, these infections were community-acquired, invasive, life-threatening ones; not infrequently with unusual locations among young, otherwise healthy individuals, i.e. showing a real, instead of opportunistic pathogenic potential of the species. The first cases were recorded in the Asian Pacific Rim including Taiwan, Korea, Vietnam and Japan. In the mid-1980s and 1990s, Taiwan has recorded patients with no previous history of hepatobiliary disease presenting with community-acquired pyogenic liver abscesses (CA-PLA) (Cheng *et al.*, 1991; Wang *et al.*, 1998; Lin *et al.*, 2018). The hvKP infections were seen in all age groups and despite the fact that some patients have co-morbidities, it is commonly noted in younger and healthy patients and as a result, hvKP will cause infection in various sites in the body such as abdominal disease, splenic abscess, thoracic disease (pneumonia), meningitis and other CNS infections, UTI and bacteremia.

1.3.2.1 Hypermucoviscous *Klebsiella pneumoniae*

Most hvKP, when growing on blood agar (BA), appear to have excess amount of mucus, even more than the regular amount causing "mucoid" appearance of the colonies of the species, i.e. these colonies are hypermucoviscous. Such strains can be identified by the 'string test'. The formation of a viscous string of > 5 mm in length using inoculation loop and stretching the bacterial colonies is considered positive as shown in Figure 4 (Shon *et al.*, 2013).

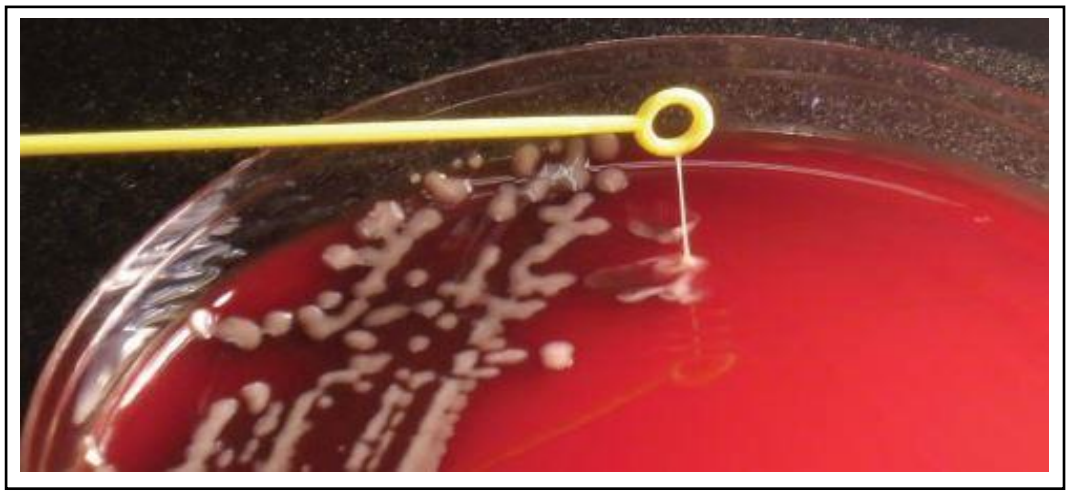

Figure 4: Positive ‘string test’ on a hypervirulent strain of Klebsiella pneumoniae (Shon et al., 2013)

1.3.2.2 Hypervirulent *Klebsiella pneumoniae*

Several genes have been implicated to contribute to the virulence of *K. pneumoniae*, like those associated with iron uptake (e.g. *iucA, iutA, ybtS, entB, kfuB*), fimbria formation (e.g. *fimH, mrkD*) and in particular the formation and hyperproduction of capsule (e.g. *magA, wabG, wcaG, rmpA, rmpA2*) (Fang *et al.*, 2007; Turton *et al.*, 2010; Candan and Aksoz, 2015). While it is still debated what are the clear criteria of hypervirulence, there are certain factors that show a close association with it.

As the initial marker of hypervirulence was linked to the highly viscous character of the strains, extensive studies were performed to identify the capsular serotypes playing unique role in facilitating it. There are nearly 80 capsular serotypes including K1, K2, K5, K16, K20, K54, K57 which were implicated to facilitate virulence in hvKP strains (Chuang *et al.*, 2006; Pan *et al.*, 2008; Cheng *et al.*, 2012). Of the different capsular serotypes, particularly K1 and K2 were the most commonly

encountered among hvKp. In one study, among the 67 *K. pneumoniae* that were isolated from the community in Canada, Denmark, Norway, South Africa, Taiwan and the United States in the period of 1996 to 2012 as 30 strains appeared to be hvKP by being hypermucoviscous. The majority (28/30) belonged to K1 capsule serotype and the other two belonged to K2 (Cheng *et al.*, 2012). In another study in China 230 strains were isolates from pneumonia, intra-abdominal infection and bloodstream infections. 37.8% of the isolates were hvKP, out of which, 33.3% were K1 and 17.2% were K2 (Wang *et al.*, 2016).

In a separate project (Yinjuan, 2017), a total of 84 hyperviscous isolates were tested for the capsular antigen and the following were identified: K1 (23.8%, 20/84), K2 (42.9%, 36/84), K5 (2.4%, 2/84), K20 (4.8%, 4/84) and K57 (9.6%, 8/84). In Singapore and Taiwan also capsular serotypes K1 and K2 dominated isolates of *K. pneumoniae* causing liver abscess. A total of 73 isolates were serotyped and the most predominant serotype K1 accounting for (34/73; 46.6%), K2 (15/73; 20.5%) and non-K1/K2 (24/73; 32.9%) (Yeh *et al.*, 2007). Through their attempt to investigate gene association with capsular serotype it was found that *magA* was restricted to, and present in all 34 K1 isolates, while all the 73 hyperviscous isolates were positive for *rmpA* (Yeh *et al.*, 2007). Severe complications of hvKP bacteremia is common. In a Chinese study (Yeh *et al.*, 2010; Hsu *et al.*, 2011). Studying 285 blood culture isolates the hypermucoviscous phenotype was seen in 24% of these isolates, the prevalence of K1 and K2 were 40.6% and 31.9%, respectively. *rmpA* and *magA* were strongly associated with viscous phenotype (Yeh *et al.*, 2010; Hsu *et al.*, 2011).

The *rmpA/rmpA2* genes (regulator of the mucoid phenotype) mediate the hyper-expression of the capsular material coding genes and can be both

chromosomally and plasmid encoded. Once on a plasmid, its size is 180–220 kb which may also code for other factors that may be involved in virulence. (Hsu *et al.*, 2011) Once again, a very close association was found between these regulators and the hvKP character (Yeh *et al.*, 2010; Hsu *et al.*, 2011).

These capsule types may also be present in strains colonizing the intestinal tract. Investigating stool specimens from 954 healthy Chinese adults who were residents of Taiwan, Hong Kong or China or were living abroad in Japan, Thailand, Malaysia, Singapore and Vietnam *K. pneumoniae* was isolated from 592 individuals and of them, 4% of the strains carried K1, while 2% expressed K2 capsular types (Brisse *et al.*, 2009). Similar results were obtained in a separate study in South Korea showing that of the 248 *K. pneumoniae* strains isolated from stool samples of healthy carriers 4.9% carried the K1 capsule type (Cheng *et al.*, 2012).

In China, (Yinjuan, 2017): nearly all (95.2%) K1, K2, K5, K20 and K57 isolates with hypermucoviscosity phenotype were positive for *rmpA*. Furthermore, this study could also show a close association between *mrkD* and the K2 capsular type isolates (Yinjuan, 2017). A similarly important finding of this study was that aerobactin-coding genes *iucA* was present in 95.1% of the hyperviscous strains and only in 41.1% of HMKP of the non-hyperviscous isolates.

Actually, the association with iron acquisition mechanisms were shown in K2 capsule type strains, too, as they produced more iron-acquisition factors than there "classic", cKP counterparts (Russo *et al.*, 2011). Indeed, and increasing body of evidences suggest that beyond surface properties, as capsular types, iron acquisition plays a central role in increasing the pathogenic potential of *K. pneumoniae* to the extreme converting the isolate to "hypervirulent". A recent study found that the

biosynthesis of siderophore aerobactin, coded by the *iuc* gene, and that of salmochelin (*iro*) are closely linked to hypervirulent *K. pneumoniae* clones causing invasive community-associated infections, such as liver abscess and pneumonia. It was also found that when examining the genome of 2503 *K. pneumoniae* isolates not known to be hypervirulent, *iuc* was detected in 8.7% and *iro* was presents in 7.2%, only (Russo *et al.*, 2015).

In another study, among *K. pneumoniae* causing severe community-acquired (CA) infections, such as pyogenic liver abscess, pneumoniae and meningitis of neonates, i.e. features of hvKP, the presence of acquired siderophores genes was also common (Holt *et al.*, 2015).

A number of other factors may also contribute to the colonization and invasion across the intestinal barrier such as the LPS, Outer Membrane Protein (OMP), genes associate with the intestinal colonization, but they seem to be less specific to the hvKP character than *rmpA/A2* and *iuc* (Struve *et al.*, 2003; Ma *et al.*, 2005; Hsieh *et al.*, 2008; Wu *et al.*, 2011). The metabolism of allantoin is a process by which bacteria can obtain carbon and nitrogen from their environment (Paczosa and Mecsas, 2016). In a search of *K. pneumoniae* genes whose transcription was upregulated in HV *K. pneumoniae* strains compared to classical strains, an operon containing genes involved in allantoin metabolism was identified. The deletion of *allS*, an activator of this operon, resulted in an HV *K. pneumoniae* strain with significantly reduced virulence in an intragastric model of infection (Chou *et al.*, 2004). A study by Yu et al., 2008, found that *allS* was present in 100% of HV *K. pneumoniae* isolates from Taiwan but it was absent in K2 isolates and non-K1/K2 isolates from patient blood or liver samples (Yu et al., 2008).

The hvKP character is, at least to some extent, associated with certain clones.

The most predominant sequence typing strongly associated with hvKP is sequence type (ST) 23 and that is linked with K1 capsular serotype and liver abscess (Pitt *et al.*, 2007; Peck *et al.*, 2008; Decre *et al.*, 2011). Furthermore, ST86, ST375 and ST380 are mainly associated with K2 serotype (Jung, 2013). A study in South Korea showed that, nearly all (94.7%) of the K1 capsule type strains (representing 4.9% of all *K. pneumoniae* isolated from fecal samples of healthy individuals) belonged to ST23 (Cheng *et al.*, 2012).

1.3.3 Bloodstream infections caused by *Klebsiella pneumoniae*

K. pneumoniae, just like *E. coli*, has been implicated in practically every kind of extraintestinal infections including urinary tract infections, tissue infections and pulmonary infections (Bennett *et al.*, 1995; Lye *et al.*, 1992; Siu *et al.*, 2012). In South America, *Klebsiella pneumoniae* represents the third most prevalent pathogen isolated from the respiratory tract of patients with pneumonia and it represents 12% of all pathogens isolated (Marra *et al.*, 2006). While these focal or more or less localized infections can have serious consequences on their own, they can also be the source of bloodstream infections (BSI) (Fader and Davis, 1980).

The status of the host defense contributes in two ways to the development of BSIs. On one hand, the initial focal infection is more likely to develop in patients with predisposing conditions and weaknesses (e.g. catheterized patients, ventilated patients, immunosuppressed patients). On the other hand, weak defenses decrease the chances to control the infection locally, and hence, increases the chances that the infection reaches the bloodstream.

Seeing an increase number of patients with decreased immunocompetence, either due to their comorbidities (e.g. malignancies) or to the medical interventions, the number of BSIs caused by *K. pneumoniae* continue to increase worldwide (Podschun *et al.,* 2001). Actually, *K. pneumoniae* considered as the second most common pathogen in the family *Enterobacteriaceae* which causes BSIs (Podschun *et al.*, 2001). Recently, international published observational study found that among the 1,156 ICU patients with BSI from all over the world 57.6% were caused by Gram-negative bacteria of which 38% was *K. pneumoniae* (Russotto *et al.,* 2015).

1.3.3.1 Bloodstream infections in patients with malignancy

Bloodstream infections (BSI) are a major cause of critical complications in patients with cancer and are directly connected with extended hospital stay, expensive healthcare costs and high risk of morbidity and mortality. The overall rate of mortality of BSI in cancer patients is reported to be 25–32%. Bacteria represent the most common cause (Obeng-Nkrumah *et al.*, 2015).

Recently, a shift from Gram-positive to Gram-negative organisms has been acknowledged in the etiology of BSI with considerable variation according to geographic location (Munoz-Price *et al.*, 2013). It has been found that Gram-negative bacteria were the most frequent contributory agents isolated in BSIs in malignant patients. It has been noticed that *K. pneumoniae*, *E. coli*, and *P. aeruginosa* are the main microorganisms involved in BSIs and are the same microorganisms which are responsible for bacteremia as reported in Europe (Lon *et al.*, 2019).

In a recent study in Mexico, it was found that 14% of patients with solid tumors developed a BSI, the ranges were from 1.2 to 1.6 cases per 1,000 patients admitted

with solid malignancies per year. In the United States, the incidence of sepsis in patients with cancer in a similar setting is about 16.4 (including hematology patients) (Lon *et al.*, 2019).

The role of the compromised defense capacities due to malignancies have been well-illustrated by a study from Taiwan. A total of 158 cases of *K. pneumoniae* bacteremia were analyzed. 40.5% of them were considered nosocomial and the most common underlying disease was neoplasia (53%), followed by hepatobiliary diseases (22%) and diabetes mellitus (n = 8,12 %) (Chang *et al.*, 2002).

The mortality rate of *K. pneumoniae* BSIs varies from 15 to 79% (Xu, *et al.*, 2018), which is lower if compared to *Acinetobacter baumannii* BSIs (30 to 84%) but higher than *Escherichia coli* (5 to 22%) (Xu *et al.*, 2018).

Beyond the actual virulence of the strains, the other major microbial factor that impacts the outcome of infections is the resistance to antibiotics (Xu *et al.*, 2018) (Kakeya *et al.*, 2016). In Italy, in a recent study on patients with haematological malignancies, of the 22 patients colonized with carbapenem resistant *K. pneumoniae* (CRKP) 14 (64%) patients developed bacteremia (Fo *et al.*, 2017). It is noteworthy due to the potentially grave consequences as BSI caused by CRKP have become a very serious problem with associated mortality as high as 40–60% (Giacobbe *et al.*, 2015).

1.4 Antibiotic resistance of *Klebsiella pneumoniae*

Antibiotics resistance, and in particular multi-drug resistance (MDR) became a severe, global burden on healthcare. The CDC identified those organisms which represent the most urgent threats of antibiotic resistance Table 3 (CDC, 2018).

Table 3: The categories of urgent threats of antibiotic resistant bacteria (CDC, 2018)

Category	Pathogens
Urgent Threats	*Clostridium difficile* Carbapenem-resistant *Enterobacteriaceae* (CRE) Drug-resistant *Neisseria gonorrhoeae*
Serious Threats	Multidrug-resistant *Acinetobacter* Drug-resistant *Campylobacter* Fluconazole-resistant *Candida* (a fungus) ESBL producing *Enterobacteriaceae* (ESBLs) Vancomycin-resistant *Enterococcus* (VRE) Multidrug-resistant *Pseudomonas aeruginosa* Drug-resistant non-typhoidal *Salmonella* Drug-resistant *Salmonella* Typhi Drug-resistant *Shigella* Methicillin-resistant *Staphylococcus aureus* (MRSA) Drug-resistant *Streptococcus pneumoniae* Drug-resistant tuberculosis
Concerning Threats	Vancomycin-resistant *Staphylococcus aureus* (VRSA) Erythromycin-resistant Group A *Streptococcus* Clindamycin-resistant Group B *Streptococcus*

It should be noted that *Enterobacteriaceae*, that includes *K. pneumoniae,* once developing resistance to the most advanced beta-lactams, i.e. carbapenems, are on the top of the list.

Antibiotics are natural products of microorganisms with the role of inhibiting the growth of other microorganisms competing for the same niche. This antimicrobial activity is being used in medicine, although in most cases the natural products are further modified, or in some cases, completely synthetized (e.g. chloramphenicol).

Antibacterial drugs can be either bactericidal or bacteriostatic depending on the species and the drug, and in some cases its concentration (Aminov, 2010). Antibiotics are grouped by their mode of action as shown in Table 4 (Murray, 2013; CDC, 2018).

Table 4: Main mode of action of antibiotics (CDC, 2018)

Mode of action	Examples
Inhibition of cell wall synthesis	ß-lactams, glycopeptides, fosfomycin
Inhibition of protein synthesis	aminoglycosides, macrolides, tetracyclines, chloramphenicol
Interference with nucleic acid synthesis	quinolones, fluoroquinolones, rifampin
Cell membrane disruption	polymyxins
Antimetabolites	sulfonamides, trimethoprim

The most common mechanisms by which bacteria could express resistance to antibiotics are the enzymatic degradation of the drug, the target alteration of its cellular target while preserving its original function, the decrease of its intercellular concentration either by decreasing its uptake or by increasing its expulsion by upregulating efflux pumps, protecting the target from the access of antibiotics and finally overproducing the target. Depending of the species and the drug, any of these mechanisms could be part of the natural or the acquired resistance mechanism of bacteria (Munita and Arias, 2016).

Acquired resistance can be the result of mutations or a cell might pick-up resistance gene(s) by taking up mobile genetic elements, such as plasmids via horizontal gene transfer (HGT) (Read and Woods, 2014; Zaman *et al.*, 2017)**.**

A broad range of antibiotics can theoretically be used to treat different *K. pneumoniae* infections, i.e. the species does not have natural resistance against them. However, experience showed that *K. pneumoniae* is particularly capable of developing

acquired resistance. Therefore, in practice, the list of drugs available to treat *K. pneumoniae* systemic and bloodstream infections (BSI) are often dangerously limited and includes ß-lactams, aminoglycosides, tigecycline, fluoroquinolones and polymyxins. Below, a brief discussion will be limited to these groups of drugs, only, together with the most common resistance mechanisms against them.

1.4.1 β-lactam antibiotics

One of the most common mechanism of antibiotic action is the inhibition of cell wall synthesis and β-lactam antibiotics (e.g. penicillins, cephalosporins, carbapenems and monobactams) are the most commonly used cell wall active antibiotics (Nordmann *et al.*, 2012). β-lactam antibiotics share a common β-lactam ring which is a core structure essential to their bacterial activity. The structures of the four groups of ß-lactam antibiotics are shown in Figure 5.

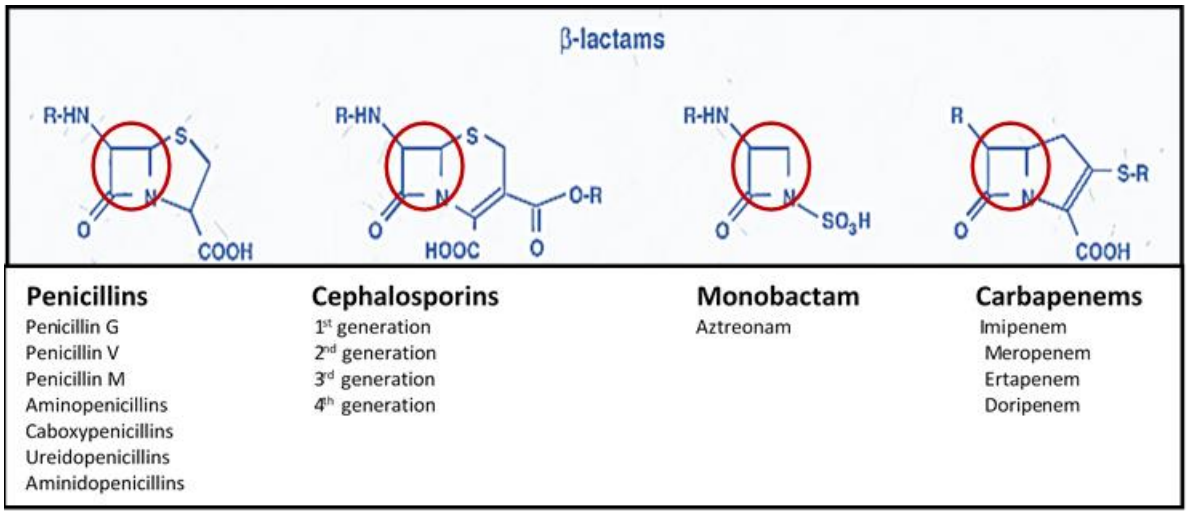

Figure 5: Chemical structures of the main β-lactams and β-lactamase inhibitors (Nordmann et al., 2012)

1.4.1.1 Penicillins

Penicillins are classified according to their spectra of antimicrobial activity. The first group includes the natural penicillin G (benzylpenicillin) and its phenoxymethyl derivative, penicillin V, both are effective against β-lactamase non-producing Gram-positive aerobic organisms including *Streptococcus*, against anaerobes, like *Clostridium* spp. and against some Gram-negative bacteria, like *Neisseria meningitidis.* Nowadays, penicillinase-resistant penicillins include methicillin, nafcillin, oxacillin, cloxacillin and dicloxacillin and they are mainly affective against *Staphylococcus* spp. that produce penicillinase. Ampicillin and amoxicillin belong to the third group called aminopenicillins and their antimicrobial activity is extended to include Gram-negative organisms, such as *Haemophilus influenzae*, *E. coli* and *Proteus mirabilis.* In the fourth group called ureidopenicillins, i.e. cabenicillin, ticarcillin, mezlocillin and piperacillin, are drugs having increased activity towards Gram-negative and anærobic organisms including *Pseudomonas* spp. (Jacoby and George, 2010; Nordmann *et al.*, 2010).

Penicillins, particularly members of the first three groups, and particularly if applied without any beta-lactamase inhibitor (see later) have very little role in treating *K. pneumoniae* infections due to their resident β-lactamase (SHV) gene (Livermore *et al.*, 2007; Sutaria *et al.*, 2018).

1.4.1.2 Cephalosporins

The cephalosporins are derived from 7-amiocephalosporanic acid as the β-lactam ring is fused with a dihydrothiazine ring (Murray, 2013). They are divided into five generations. Through generations 1-3 the spectrum of cephalosporins has

gradually drifted towards Gram-negative cells with somewhat decreasing activity against Gram-positive organisms. The 3rd and 4th generation cephalosporins are extensively used for the treatment of *K. pneumoniae* infections (Cole *et al.*, 2015; Lee *et al.*, 2015)**.** The 5th generation drugs are active against MRSA due to their increased affinity to PBPs.

The classification of cephalosporins generations in Table 5 is based on the general features of antimicrobial activity (Dancer, 2001).

Table 5: The generations of cephalosporins

Generations	Spectrum
First Generation: Cefazolin Cephalexin monhydrate Cefadroxil Cephradine	*Streptococci, Staphylococcus aureus*
Second Generation: Cefuroxime Cefuroxime axetil Cefprozil Cefmetazole Loracarbef	*Escherichia coli, Klebsiella, Proteus, Haemophilius influenzae, Moraxella catarrhalis*. Not as active against gram-positve organisms as first-generation agents. Inferrior activity against *S. aureus* compared to cefuroxime but with added activity against *Bacteroides fragilis* and other *Bacteroides spp.*
Third Generation: Cefotaxime Ceftriaxone Cefdinir Cefditoren pivoxil Ceftibuten Cefpodoxime proxetil Ceftizoxime Cefoperazone Ceftazidime	*Enterobacteriaceae, Pseudomonas aeruginosa, Serratia, Neisseria gonorrhoeae,* activity for *S. aureus*, *Streptococcus pneumoniae,* and *Streptococcus pyogenes* comparable to first-generation agents. Activity against *Bacteroides spp*. inferior to that of cefoxitin and cefotetan Active against Pseudomonas
Fourth Generation: Cefepime	Comparable to third generation but resistant to Beta-lactamase
Fifth Generation: Ceftobiprole Ceftaroline	Active against MRSA Not resistant to ESBL

1.4.1.3 Monobactams

Aztreonam is the main representative of monobactams. They have a narrow spectrum activity against aerobic Gram-negative microorganisms without any activity against Gram-positive bacteria. While having not been used extensively, lately there are likely to experience an increased importance of aztreonam, as this is the only group of beta-lactams that are resistant to metallo-beta-lactamases (MBL) (Vardanyan and Hruby, 2016).

1.4.1.4 Carbapenems

Members of this group (e.g. imipenem, meropenem, ertapenem and doripenem) have the broadest spectrum of all ß-lactams against both Gram-positive and Gram-negative organisms. Importantly, non-fermenting bacteria (e.g. *Acinetobacter* and *Pseudomonas spp.*) have natural resistance against ertapenem. Many MDR Gram-negative strains are still sensitive to carbapenems and hence, today, they are the ultimate drugs used to treat various life threatining Gram-negative infections. (Bush and Karen, 2007; Papp-Wallace *et al.*, 2011). That is why the fast emergence and spread of carbapenem resistance in *Enterobacteriaceae* (CRE) and that in *K. pneumoniae* (CRKP) is such a serious threat (CDC, 2018).

1.4.2 Resistance to β-lactams antibiotics

There are three main mechanisms by which bacteria can resist β-lactams: target modification, decreased intracellular concentration and by the production of beta-lactamase enzymes.

1.4.2.1 Target alteration

This is a common and highly effective mechanism in several Gram-positive bacteria (*e.g.* in methicillin resistant *Staphylococcus aureus,* MRSA or in *Streptoccoccus pneumoniae*), and is also present in some Gram-negative ones (*e.g.* the ß-lactamase negative, ampicillin resistant (BLNAR) strains of *Haemophilus influenzae* (Dever and Dermody, 1991; Lambert, 2005; Tristram *et al.*, 2007). However, in converting *Enterobacteriaceae* to ß-lactam resistant it has limited, if any role, hence it will not be further discussed here.

1.4.2.2 Decreasing intracellular concentration

The intracellular concentration of a drug can be limited by two ways: by expelling molecules already taken up or by limiting their uptake. The first mechanism is usually achieved by the up regulation of the expression of efflux pumps (Poole, 2005; Webber and Piddock, 2003). These efflux pumps vary in their spectrum of compounds as they can expel from the cell, but often they are multi-drug efflux pumps (Aeschlimann, 2003; Rouveix, 2007; Sun *et al.*, 2014). Although this spectrum may include various ß-lactam drugs in *Enterobacteriaceae,* in their cephalosporin and carbapenem resistance efflux pumps play a comparatively minor role, seldom leading to clinically significant level of resistance to these agents (Pages *et al.*, 2009).

The other mechanism is the modification of porins that form channels in the outer membrane or limiting their numbers with a consequent limited uptake of the drugs (Yang *et al.*, 2010; Nikaido, 1989; Doumith *et al.*, 2009). This mechanism has considerable effectiveness in *Enterobacteriaceae* in lowering the susceptibility to cephalosporins and carbapenems (Pages *et al.*, 2008; Wozniak *et al.*, 2012). Even if

the restriction of uptake may not result in clinically significant reduction of susceptibility, once combined with the production of hydrolytic enzymes even with moderate activity against a particular extended spectrum beta-lactam, the combined effect of these two mechanisms can cause clinically significant cephalosporin or even carbapenem resistance.

1.4.2.3 Enzymatic degradation of β-lactams

Enzymatic inactivation of β-lactam agents is the most common and most effective resistance mechanism among *Enterobacteriaceae* against this group of antibiotics (Murray, 2013).

There are two main classification schemes for ß-lactamases that are currently in use. The Ambler molecular system, based on conserved and distinguishing amino acid motifs, classifies β-lactamases into four molecular classes, *i.e.* A, B, C, and D, (Table 6)**.** On the other hand, grouping by the Bush-Jacoby-Medeiros system is based on the functional similarities (including substrate spectrum, EDTA and susceptibility to inhibitors, such as clavulanic acid, sulbactam and tazobactam) of the various enzymes forming four main, and multiple subgroups (Table 6) (Ambler, 1980; Bush and Jacoby, 2010; Hall and Barlow, 2005).

Table 6: Classification of β-lactamases (Bush and Jacoby, 2010; Bush et al., 1995)

Molecular class	Bush-Jacoby-Medeiros	Active site	Enzyme type	Substrates	Examples	Inhibitors	Comment
A	2a 2b 2be 2br 2c 2e 2f	Serine	Penicillinases Broad Spectrum	Aminopenicillins	TEM1 SHV1	Clavulanic acid	Plasmid Chromosome
			Extended Spectrum ß-lactamases (ESBL)	Broad spectrum and aztroneam	TEM-derived, CTX-derived	Clavulanic acid	Plasmid
			Carbapenemases	Extended spectrum plus cephamycins	KPC-2 KPC-3	Clavulanic acid	Chromosome
B	3	Zn^{2+}	Metallo-β-lactamases	Extended spectrum plus cephamycins	IMP, VIM, GIM, NDM	EDTA	Plasmid Chromosome
C	1 1e	Serine	Cephalosporinases	Extended spectrum plus cephamycins	AmpC-type	Cloxacillin Monobactam	Plasmid Chromosome
D	2d	Serine	Oxacillinases Broad spectrum	Cloxacillin, methicillin	OXA-family in *P. aeruginosa*	Clavulanic acid +/-	Plasmid Chromosome
			Extended spectrum	Broad spectrum and monobactams	OXA-derived in *P. aeruginosa*	Clavulanic acid +/-	Plasmid Chromosome
			Carbapenemases	Extended spectrum plus cephamycins	OXA-derived in *Acinetobacter*	Clavulanic acid +/-	Plasmid Chromosome

Class A contains beta-lactamases with a broadly varying substrate specificity from narrow spectrum penicillinases through extended-spectrum beta-lactamases (EBLS) hydrolyzing 3rd and 4th generation cephalosporins to carbapenemases. Several, but not all of them can characteristically be inhibited by β-lactamase inhibitors (see later). Their genes are often located on plasmids; therefor they have the ability to spread among members of the same species or even among different genera.

The best-known representatives of this group are TEM and SHV type enzymes. The original molecules had relative narrow substrate spectrum. However, subsequent mutations have resulted into the considerable extension of the spectrum also including 3rd and 4th generation cephalosporins (and in some case a low activity also against carbapenems) (Fong and Drlica, 2003; Harvey *et al.*, 2007; Rupp and Fey, 2003). Currently, the CTX-M type of ESBLs, with characteristically high activity against cefotaxime, represent a rapidly growing family of molecular class A ESBLs. In recent years, the CTX-M type ESBLs became the most frequent ESBL type worldwide (Bonnet, 2004; Cantón *et al.*, 2008; Eisner *et al.*, 2006; Zowawi *et al.*, 2013).

Further members of this group are the enzymes with even broader activity spectrum, *i.e.* carbapenemases. An important example is the *K. pneumoniae* carbapenemases (KPC), first detected in USA in 1996, that is currently the most common carbapenemases at several parts of the World (Arnold *et al.*, 2011; Nordmann *et al.*, 2009; Sonnevend *et al.*, 2015a; Won *et al.*, 2011; Zowawi *et al.*, 2014).

Molecular class B enzymes are the so-called metallo ß-lactamases (MBL) that require a divalent cation for their activity and hence, can be inactivated by chelators, such as EDTA or dipicolinic acid (DPA). There are several broad-spectrum enzymes among them with activity against all ß-lactams (including carbapenems) with the

notable exception of monobactams. Similarly, to group A enzymes, their genes can often be found on mobile genetic elements. They are commonly present in non-fermenting bacteria, such as *Pseudomonas* and *Acinetobacter*¸ but also spreading fast among *Enterobacteriaceae*. Examples of these enzymes are the VIM, IMP and the recently emerging New Delhi Metallo ß-lactamases (NDM) (Cornaglia *et al.*, 2011; Ghazawi *et al.*, 2012; Sonnevend *et al.*, 2012; Walsh and Toleman, 2012).

The most important types of class C enzymes are the AmpC-type enzymes. Some, if hyper-expressed, has broad spectrum activity which includes hydrolysis of 3rd and 4th generation cephalosporins, and in some cases have even a moderate, weak activity against carbapenems. In some species the necessary high expression of the enzyme to achieve clinical resistance is inducible by ß-lactam drugs, while in others they are constitutively expressed (Hanson, 2003; Jacoby, 2009; Thomson, 2010).

Molecular class D enzymes are primarily oxacillinases (OXA) that vary considerably in their spectrum. An important group even has carbapenemase activity, although some of these enzymes exhibit simultaneously no or moderate activity against cephalosporins (Evans and Amyes, 2014; Walther-Rasmussen and Høiby, 2006). They predominantly occur in various non-fermenting Gram-negative rods, but currently a subset of them, the OXA-48-like enzymes, are fast spreading in *Enterobacteriaceae,* as well (Ahn *et al.*, 2015; Cantón *et al.*, 2012).

1.4.3 Beta-lactamase inhibitors

Beta-lactamase inhibitors (Figure 6) are the most powerful and clinically used antibiotic adjuvants to counteract resistance to beta-lactam antibiotics. The main role of these inhibitors is to protect the antibiotics from a very efficient bacterial inactivation mechanism involving beta-lactamases (González-Bello, 2017).

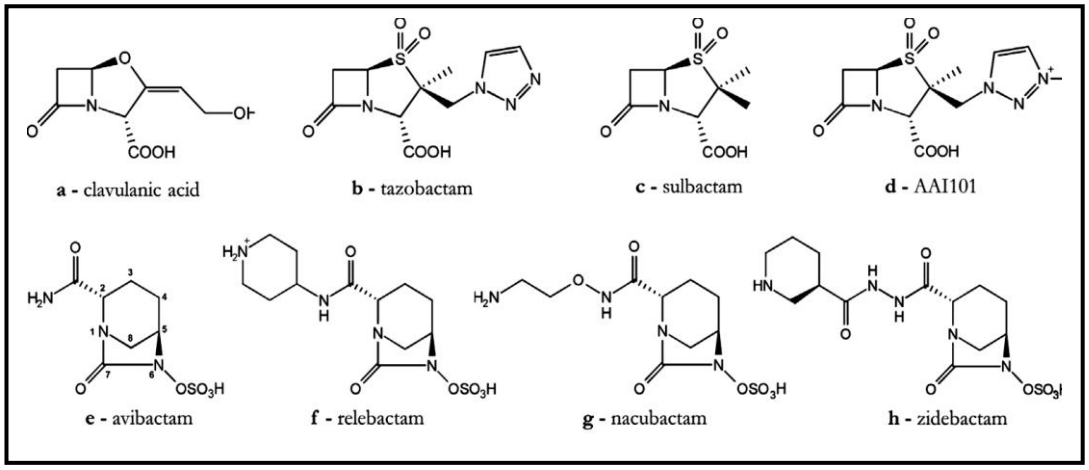

Figure 6: Chemical structures of β-lactamase inhibitors (Docquier and Mangani, 2018)

The identification of inhibitors to common beta-lactamases started in the mid-1970s, which was due to the appearance of the TEM-1 penicillinase in *Neisseria gonorrhoeae* (Ashford *et al.*, 1976) and *Haemophilus influenzae* (Gunn *et al.*, 1974; Khan *et al.*, 1974). The first inhibitor discovered was clavulanic acid with a novel clavam structure with an inhibitor activity against staphylococcal penicillinases and most of the known plasmid-encoded penicillinases found in enteric bacteria (Reading and Cole 1977; Cole, 1982), including the widespread TEM and SHV enzymes (Simpson *et al.*, 1980). Clavulanic acid is a so called “suicide inhibitor” as it is being inactivated while interfering with the enzyme to be inhibited (Charnas *et al.*, 1978; Charnas and Knowles, 1981).

Several, but not all, class A beta-lactamases, including ESBLs, can be inhibited by clavulanic acid (Steward *et al.*, 2001) and that applies to some extent even to some of the class A carbapenemases (Nordmann and Poirel 2002; Yigit *et al.*, 2003). Most extensively it is used in combination with amoxicillin considerably extending the spectrum of the drug particularly towards Gram-negative bacteria (Bush and Bradford, 2016).

After the discovery of clavulanic acid, chemists tried to synthesize a number of penicillanic acid sulfones with beta-lactamase inhibitory activity (English *et al.*, 1978; Fisher *et al.*, 1981; Aronoff *et al.*, 1984). Of these, sulbactam (English *et al.*, 1978) and tazobactam (Aronoff *et al.*, 1984) were effectively commercialized. These two inhibitors had a similar range of activity as clavulanic acid. Sulbactam has somewhat less inhibitory activity against class A beta-lactamases compared to clavulanic acid or tazobactam but has an improved inhibition against class C cephalosporinase enzimes (Bush *et al.*, 1993). The sulfone inhibitors do not work as inducers of chromosomally mediated AmpC Beta-lactamase (Weber and Sanders 1990). Sulbactam has been combined with ampicillin (Neu, 1990) and in Japan it has been combined with cefoperazone to offer extra synergistic activity against non-fermentative and anaerobic bacteria (Eliopoulos *et al.*, 1989). On the other hand, tazobactam has been combined with piperacillin, cefoperazone and ceftolozane for nosocomial infections, including those caused by *P. aeruginosa* (Lister, 2000).

Despite the structural similarities to “real” beta-lactam drugs, the inhibitors usually have limited antibacterial activity on their own with some exceptions. Clavulanic acid alone has an MIC as low as 1 mg/L against *N. gonorrhoeae* (Wise *et al.*, 1978); while sulbactam has intermediate activity against wild-type *Acinetobacter*

spp. and *Burkholderia cepacea*, respectively (Jacoby and Sutton 1989; Fass *et al.*, 1990; Dong *et al.* 2014). Importantly, none of these inhibitors inhibit the hydrolytic activity of MBLs (Bush, 2015), and that their activity against serine carbapenemases, if present at all, is not sufficient to convert enzyme producing isolates into clinical susceptibility (Yigit *et al.*, 2003; Woodford *et al.*, 2004; Moland *et al.*, 2007).

After a gap of almost two decades, a new class of non-beta-lactam beta-lactamase inhibitors emerged based on a novel structure, i.e., diazabicyclooctane (DBO) (Coleman, 2011). The first inhibitor of this class is Avibactam which has a broader spectrum of activity than the previous inhibitors.

The success of avibactam can be attributed first to the unique structural similarity to beta-lactams at the level of the electrophilic carbonyl group. This mimicry is significant for rapid formation of a stable adduct by beta-lactamases. The stable acyl enzyme formed by the carbamoyl link between the inhibitor and the enzyme active-site serine residue considered the second significant attribute of this compound (Drawz *et al.*, 2014).

Avibactam can effectively inhibit not only class A penicillinases, ESBLs, and serine carbapenemases, but also class C cephalosporinases and some class D oxacillinases (Ehmann *et al.*, 2012, 2013). It does not induce AmpC beta-lactamases at clinically applicable concentrations (Coleman, 2011). Avibactam, in combination with ceftazidime, has been approved in late 2014 by the FDA to treat complicated intra-abdominal and complicated urinary tract infections (González-Bello, 2017).

Some other DBOs which are under development include RG6080 and relebactam (MK7655), in combination with imipenem-cilastatin. The inhibitory

spectrum of relebactam is similar to that of avibactam; however, except that it does not inhibit class D beta-lactamases, such as OXA-48 (Livermore *et al.*, 2013). The other inhibitor RG6080 (earlier known as OP0565) is a DBO that has similar inhibitory spectrum to the other DBOs, but it has the extra benefit of showing some natural antibacterial activity against enteric bacteria (Livermore *et al.*, 2015). The RPX7009 (boronic acid) inhibitor is another new class of synthetic non-b-lactam b-lactamase inhibitors (Hecker *et al.*, 2015). It has inhibitory activity against many groups of serine beta-lactamases (Hecker *et al.*, 2015).

None of these promising inhibitors has activity against class B enzymes. However, clinical trials using aztreonam in combination with ceftazidime are promising. MBLs do not hydrolyze aztreonam and the avibactam added may inhibit practically all, non MBL type beta-lactamases. (Drawz *et al.*, 2014).

1.4.4 Aminoglycosides

The discovery of aminoglycosides through systematic screening of soil *Actinobacteria* started in the 1940s. Streptomycin was the first aminoglycoside to be discovered from *Streptomyces griseus* and since then, it has been successfully used for the treatment of tuberculosis and infections with Gram-negative bacteria (Doi *et al.*, 2016). The name “aminoglycoside” derives from the classic structure of these antibiotics having an amino-containing or non-amino-containing sugar residue connected to six-membered rings with amino group substituents. Subsequently, numerous aminoglycosides (Figure 7) have been identified or semi-synthesized and used in clinical practice. Based on their chemical structures, the aminoglycosides are grouped into 4,6-disubstituted 2-deoxystreptamine (DOS), 4,5-disubstituted DOS, and 4-monosubstituted DOS.

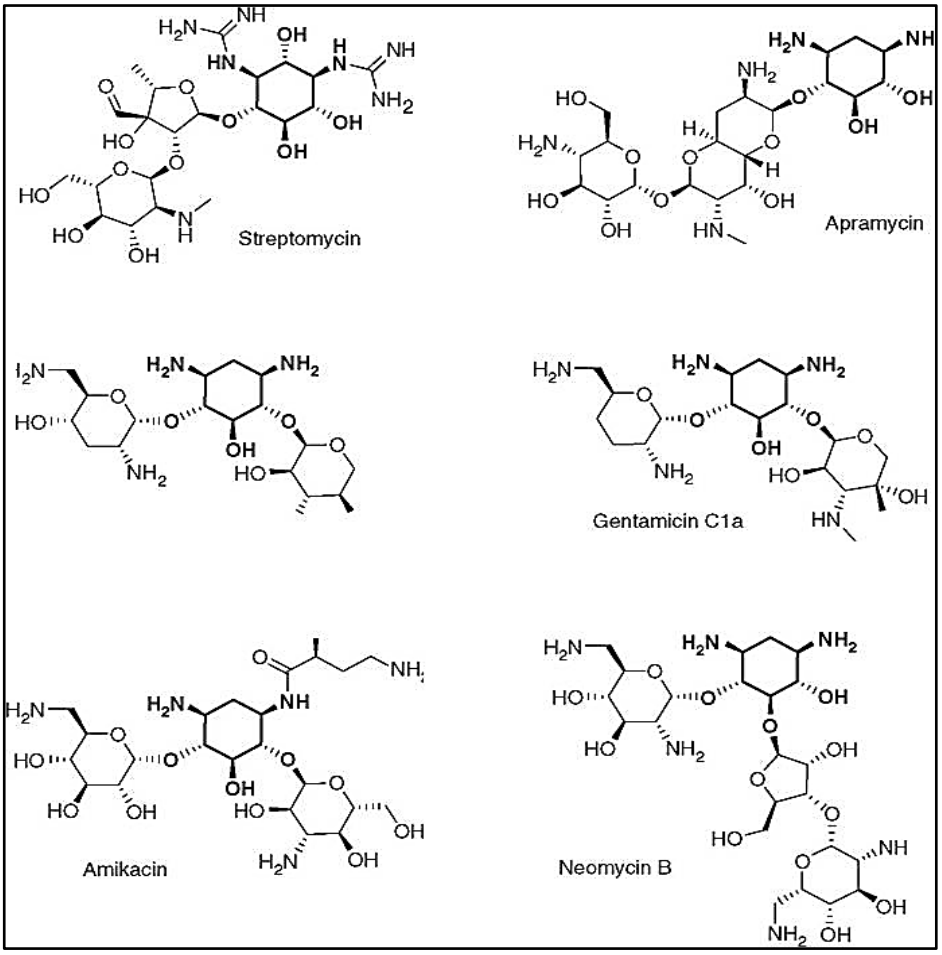

Figure 7: Structures of representative aminoglycosides (Krause et al., 2016)

The first group includes gentamicin, tobramycin, and amikacin, which have been extensively used as intravenous or nebulized formulations in combination with a beta-lactam for the treatment of infections caused by Gram-negative bacteria, and atypical mycobacteria (also in combination with other active agents). The second group is represented by neomycin, which are limited in their use for being toxic and are given either orally or topically, but not intravenously. The monosubstituted DOS group is represented by apramycin, which is mainly used in veterinary medicine (Doi *et al.*, 2016). Lately, a new semisynthetic version, plazomicin, was introduced to clinical practice, that is able to resist the most aminoglycoside destroying mechanisms (Petrosillo *et al.*, 2019).

Aminoglycosides lead to inhibition of polypeptide synthesis and to subsequent cell death by binding to the aminoacyl-tRNA recognition site (A-site) of the 16S rRNA which constitutes the 30S ribosomal subunit. They are the only protein synthesis inhibitor antibiotics with a bactericidal effect against most susceptible pathogens (Doi *et al.*, 2016; Serio *et al.*, 2018).

1.4.4.1 Resistance to aminoglycosides

There are several mechanisms by which bacteria can confer resistance to aminoglycosides of which: (1) enzymatic modification and inactivation of the aminoglycosides, mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferases (Ramirez and Tolmasky, 2010; Shaw *et al.*, 1993); (2) increased efflux; (3) decreased permeability; and (4) modifications of the 30S ribosomal subunit; (5) target site modification which occurs through the action of 16S rRNA methyltransferases (RMTs). Mechanisms 1-4. have varying effects on different members of the class and frequently multiple mechanisms are involved in any given resistant isolate. Nevertheless, by choosing the right representative, often there is a chance to find a drug that is still clinically active. On the other hand, modification of the 16S ribosomal site usually affects most clinically relevant aminoglycosides (Krause *et al.*, 2016; Takahashi and Igarashi., 2018) seriously restricting the use of these antibiotics. Hence, in the following paragraph, the focus will be on the 16S RMTs.

RMTs consist of two main classes depending on the specific nucleotide residues that they modify. The first class includes enzymes that make the bacteria resistant to 4,6-di-substituted aminoglycosides via methylation of the N7 position of nucleotide G1405 (Thompson *et al.*, 1985; Beauclerk and Cundliffe 1987) while the

other class affects both 4,6- and 4,5-di-substituted aminoglycosides through methylation of the N1 position of nucleotide A1408 (Skeggs *et al.*, 1985; Beauclerk and Cundliffe 1987; Mingeot-Leclercq *et al.*, 1999).

The first RMT described was *armA* in *Klebsiella pneumoniae* and *rmtA* in *Pseudomonas aeruginosa* (Galimand *et al.*, 2003; Yokoyama *et al.*, 2003). The *armA* gene was first reported in an isolate of *K. pneumoniae* from the urine of a patient in Paris in 2000 (Galimand *et al.*, 2003; Gniadkowski *et al.*, 1998). *armA* was later confirmed to function as N7- G1405 16S-RMTase (Liou *et al.*, 2006). *rmtA* was identified in a sputum isolate of *P. aeruginosa* in Japan in 1997 (Yokoyama *et al.*, 2003). Since then several others were described (Table 7) (Doi *et al.*, 2016).

Of the enzymes methylating at N1 A1408 position only a single representative has so far been described: *npmA*, was reported from an *Escherichia coli* clinical strain in Japan in 2007 (Wachino *et al.*, 2007). It confers an even wider spectrum of aminoglycoside resistance than the N7 methylating ones including neomycin and apramycin (Dunkle *et al.*, 2014). Importantly, several of these genes are located on plasmids, often also carrying other resistance genes (Doi *et al.*, 2016).

Table 7: Acquired 16S-RMTases (Doi et al., 2016)

16S-RMTase	Common Species	Common Co-resistance	Prevalence	Distribution
armA	*K. pneumoniae* *A. baumannii*	CTX-M ESBL, NDM carbapenemase, OXA-23 carbapenemase	Very high in A baumannii High among NDM producers	Worldwide
rmtA	*P. aeruginosa*	—	Low	Japan, Korea
rmtB	*E. coli* *K. pneumoniae*	CTX-M ESBL, NDM carbapenemase	High in China High among NDM producers	Worldwide
rmtC	*K. pneumoniae* *P. mirabilis*	NDM carbapenemase	High among NDM producers	India, United Kingdom
rmtD	*P. aeruginosa* *K. pneumoniae*	CTX-M ESBL, KPC carbapenemase	Low	South America
rmtE	*E. coli*	CMY-2 AmpC	Very low	United States
rmtF	*K. pneumoniae*	NDM carbapenemase	High among NDM producers	India, United Kingdom
rmtG	*K. pneumoniae*	CTX-M ESBL, KPC carbapenemase	Low	South America
rmtH	*K. pneumoniae*	CTX-M ESBL	Very low	Iraq
npmA	*E. coli*, *K. pneumoniae* *Enterobacter spp.*	—	Very low	Japan, Saudi Arabia

1.4.5 Tigecycline

Tigecycline is a glycylcycline antimicrobial agent that is structurally related to minocycline (Sun *et al.*, 2013). Tigecycline can enter the bacterial cell by active transport or passive diffusion and, in the same way as tetracyclines, the antibiotic binds reversibly to the 30S ribosomal subunit, hindering the entry of tRNA into the A site of the ribosome therefore preventing the elongation of peptide chains and thus limiting

the bacterial growth (Noskin, 2005; Hirata *et al.*, 2004; Hope *et al.*, 2010; Khardori, 2006). The addition of an N,N,-dimethylglycylamido group at the 9 position of the minocycline molecule increases the affinity of tigecycline for the ribosomal target up to 5 times when compared with minocycline or tetracycline (Nathwani, 2005).

Tigecycline has a broad range of activity against many Gram-positive (such as MRSA and MRSE, penicillin-resistant *Streptococcus pneumoniae*, and VRE species), gram-negative (*Citrobacter freundii*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella* species, *Salmonella* species, *Serratia marcescens*, and *Shigella* species) and anaerobic organisms (Zhanel *et al.*, 2004; Nathwani, 2005; Fritsche and Jone, 2004). Activity of tigecycline and minocycline can also cover *Acinetobacter* species (Pachon-Ibanez *et al.*, 2004). None of the tetracyclines have coverage against *Pseudomonas aeruginosa*. Tigecycline also does not have activity against certain Proteus strains, including *Proteus mirabilis*. A major disadvantage of tigecycline is its short half live in serum, so its main use is in soft tissue infections intrabdominal and lately, pneumonias (Stein and Babinchak, 2013; Wang et al., 2017).

1.4.5.1 Resistance to tigecycline

It has been reported that tigecycline is not affected by most of the common mechanisms of antibiotic resistance used by bacteria to avoid antibiotic therapy, due to the steric hindrance gained by the large D-ring substituent (Pankey, 2005). However, emergence of tigecycline resistance in *K. pneumoniae* isolates has been reported (Elgendy *et al.*, 2018). Overexpression of *rarA*, a transcriptional activator of the *oqxAB* efflux pump, can induce a low level of resistance to tigecycline in *K. pneumoniae* (Elgendy *et al.*, 2018).

Based on large-scale surveillance studies, tigecycline appears to hold low resistance rates of <10% among *Enterobacteriaceae* worldwide (Pournaras *et al.*, 2016). An overall non-susceptibility rate of <10% including ESBL-producing *Enterobacteriaceae* and CR *K. pneumoniae*, and <20% for MDR *Enterobacteriaceae* and CR *Enterobacter* spp. and *S. marcescens* was reported (Pournaras *et al.*, 2016). On a worldwide basis, it has been found that *E. coli* exhibits lower non-susceptibility rates if compared to *K. pneumoniae*, *E. aerogenes*, *E. cloacae* and *S. marcescens* (Fern´andez *et al.*, 2012). Using next-generation sequencing, Zheng et al identified several genes which probably encode efflux pumps or translational regulators in tigecycline-resistant *K. pneumoniae*, based on their sequence similarity to known RND efflux pump genes which can be related to tigecycline resistance (Zheng *et al.*, 2014). It is clear that Tigecycline resistance mechanisms are complex in *Enterobacteriaceae* (Table 8) (Zheng *et al.*, 2014; Wang *et al.*, 2015; Pournaras *et al.*, 2016).

Table 8: Mechanisms implicated in tigecycline resistance among Enterobacteriaceae (Pournaras et al., 2016)

Pathogen	Suggested mechanism of tigecycline resistance
Klebsiella pneumonia	Overexpression of *ramA* and *acrAB*
	Deletions, insertions and point mutations in *ramR*
	Overexpression of *rarA* and *oqxB*
	IS*5* element integration in a putative efflux pump, KpgABC
	Overexpression of *marA* and *acrAB*
	Structural alteration of the ribosomal protein S10
	Other RND efflux pump genes
Enterobacter cloacae and *Enterobacter aerogenes*	RamA- mediated mechanisms affecting AcrAB regulation
Escherichia coli	AcrAB and AcrEF RND efflux pumps
	Single nucleotide insertion in *marR*/ overexpression of MarA
Proteus mirabilis	AcrAB efflux pump
Morganella morganii	AcrAB efflux pump
Serratia marcescens	Upregulation of SdeXY–HasF efflux pump

IS, insertion sequence; RND, resistance–nodulation–cell division.

1.4.6 Fluoroquinolones

The quinolones are a group of synthetic antimicrobial agents with major clinical relevance. They have been one of the most frequently prescribed antimicrobial

agents in the world. Initially, they were mostly used to treat Gram-negative infections, later they were modified in order to improve their pharmacokinetic properties and extend their antibacterial spectrum, to become effective against a wide variety of Gram-negative and Gram-positive pathogens (Correia *et al.*, 2017).

Nalidixic acid, as the first compound of the quinolones, was introduced into clinical use in 1962, for the treatment of uncomplicated urinary tract infections (UTIs) (Fàbrega *et al.*, 2009; Bisacchi, 2015). The second generation of compounds, the fluoroquinolones, showed significantly improved activity and enhanced Gram-positive penetration and better pharmacokinetic and pharmacodynamic properties (Aldred *et al.*, 2014). The first representative of this generation was norfloxacin but ciprofloxacin, the most widely used representative, showed good activity also outside the urinary tract (Bisacchi, 2015). Even after almost three decades in clinical use, the World Health Organization (WHO) listed ciprofloxacin as an essential, critically important antibiotic (Collignon *et al.*, 2016). The huge success of ciprofloxacin led to the development of a newer generation of quinolones (levofloxacin, moxifloxacin, gatifloxacin, etc.) with broader and different spectrum of activity and pharmacokinetic properties (Aldred *et al.*, 2014). Fluoroquinolones have been used comprehensively for multiple clinical indications worldwide due to their power, wide activity spectrum, oral bioavailability and generally good safety profile (Hooper and Jacoby, 2016).

The quinolones are known to target and inhibit the activity of two necessary bacterial type II topoisomerases: DNA gyrase and topoisomerase IV, respectively which play a vital role in the modulation of the chromosomal supercoiling required for DNA synthesis, transcription and cell division (Aldred *et al.*, 2014; Kohanski *et al.*, 2010).

The number of quinolone-resistant strains has been growing steadily, being observed in all species exposed to this antimicrobial class (Aldred *et al.*, 2014; WHO, 2014; Hooper and Jacoby, 2016).

1.4.6.1 Resistance to fluoroquinolones

Quinolone resistance can be due to several mechanisms (Figure 8). Chromosomally encoded mechanism are mutations modifying the target enzymes and their drug-binding affinity and those leading to reduced drug accumulation by either decreased uptake or increased efflux. Plasmid-coded resistance can be due to the production of proteins protecting the drug's and to the expression of drug modifying enzymes (Aldred *et al.*, 2014; Hooper and Jacoby, 2016; Kim and Hooper, 2014). These mechanisms often co-exist and act concomitantly leading to high levels of quinolone resistance (Hopkins *et al.*, 2005; Hooper and Jacoby, 2016).

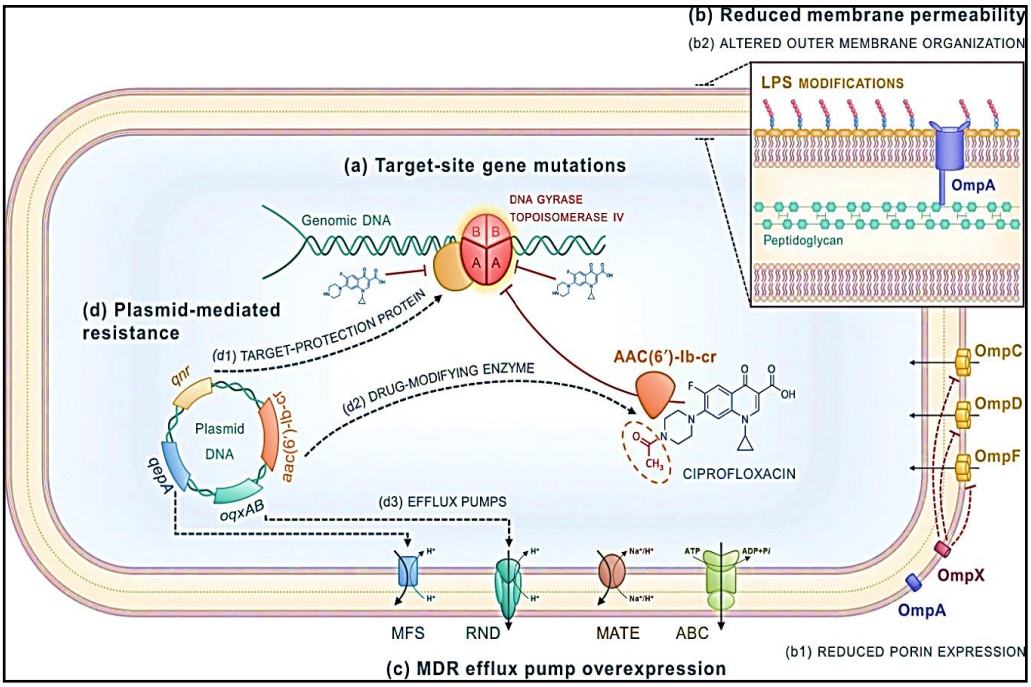

Figure 8: Mechanisms of quinolone resistance (Correia et al., 2017)

Plasmid-mediated quinolone resistance (PMQR) is of particular importance due to the easy spread via conjugation. The first target-protecting mechanism was described in the late 1990s and have been broadly reviewed in the last decade (Jacoby *et al.*, 2014; Rodríguez-Martínez *et al.*, 2016; Ruiz *et al.*, 2012). The first PMQR gene, *qnrA*, was reported in 1998 in a conjugative plasmid of a ciprofloxacin-resistant *K. pneumoniae* clinical isolate (Martínez-Martínez *et al.*, 1998) and, nowadays, about 100 Qnr variants have been identified, which has been classified into six distinct families: QnrA, QnrB, QnrS, QnrC, QnrD and QnrVC. It has been found that the Qnr proteins belong to the pentapeptide-repeat protein family and provide quinolone resistance by physically protecting DNA gyrase and topoisomerase IV from quinolone inhibition (Correia *et al.*, 2017).

The second mechanism for PMQR was discovered in 2006 as a bifunctional variant of an aminoglycoside-modifying acetyltransferase, AAC(6')- Ib-cr, that has two specific amino acid substitutions (Trp102Arg and Asp179Tyr). This variation made it able to acetylate the unsubstituted nitrogen of the C7 piperazine ring, which is present in quinolones such as ciprofloxacin, thus conferring quinolone resistance by decreasing the drug's activity. Soon after, a third mechanism for PMQR was found with the discovery of the plasmid- encoded efflux pumps QepA and OqxAB (Correia *et al.*, 2017).

In the presence of therapeutic levels of quinolones, PMQR provides a good background for selection of additional resistance mechanisms and hence for the emergence of high levels of quinolone resistance. It has been reported that PMQR genes found in transposons and/or integrons that are often co-located with other resistance determinants (frequently with extended spectrum B-lactamase, AmpC-type

B-lactamase and carbapenemase genes) in MDR plasmids of significantly varying sizes and incompatibility groups. The circulation of multiple plasmids with such heterogeneity has been accountable for the wide geographic distribution of PMQR in a great variety of hosts (Correia *et al.*, 2017).

1.4.7 Polymyxins

In an era of enormous and growing threat of increased bacterial resistance to almost all available antibiotics, drugs, such as the polymyxins, previously confined to topical use, only due to their toxicity are re-entering the therapeutic armory against Gram-negative bacteria (Yu *et al.*, 2015; Poirel *et al.*, 2017). Consequently, polymyxins are often considered as the last option of antibiotic therapy for MDR bacteria (Rice, 2006; Li and Nation, 2006).

The polymyxins consist of polymyxins A till E, of which only polymyxin B and polymyxin E (colistin) are now used clinically. In general, both have a narrow antibacterial spectrum restricted to Gram-negative bacteria (Li *et al.*, 2007). Most members of the *Enterobacteriaceae* family, including *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp.) and the common non-fermenters (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*) are naturally susceptible (Falagas and Kasiakou, 2005). Some species are naturally resistant to polymyxins, including *Proteus* spp., *Morganella morganii*, *Providencia* spp., *Serratia marcescens.* Polymyxins are not active against Gram-negative cocci (*Neisseria* spp.), Gram-positive bacteria, and anaerobic bacteria (Falagas and Kasiakou, 2005).

Colistin (also known as polymyxin E) was originally isolated in 1947 from the

soil bacterium *Paenibacillus polymyxa* subsp. *colistinus* (Benedict and Langlykke, 1947; Biswas *et al.*, 2012). In the 1980s, the parenteral use of polymyxin was almost completely abandoned due to the emergence of less-toxic aminoglycosides and other antipseudomonal agents (Poudyal, *et al.*, 2008).

Polymyxins are cationic polypeptides that contains a cyclic heptapeptide possessing a tripeptide side chain acylated at the N terminus by a fatty acid tail (Falagas *et al.*, 2010; Li *et al.*, 2006) (Figure 9).

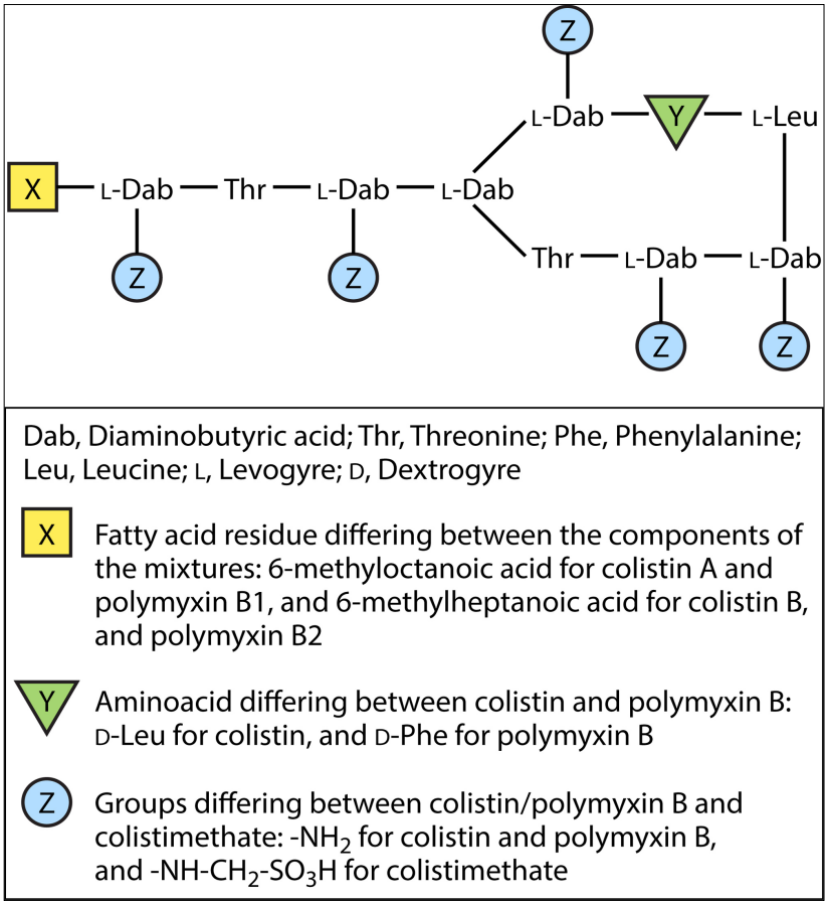

Figure 9: Structures of colistin A and B, colistimethate A and B, and polymyxin B1 and B2 (Poirel et al., 2017).

The intrinsic toxicity of colistin may be due to the hydrophobic properties of the N-terminal fatty acyl segment, which also play a significant role for its antimicrobial activity, and also by positions 6 and 7, which are very important (Brink *et al.*, 2014; Gallardo-Godoy *et al.*, 2016). Colistin and polymyxin B are different by just a single amino acid in the peptide ring (Figure 9) (Nation *et al.*, 2014). While the polymyxin B is administered directly as an active antibiotic, the colistin must be administered as an inactive prodrug, colistin methanesulfonate (colistimethate [CMS]) (Nation *et al.*, 2014).

The cationic polymyxins bind to the anionic outer membrane of Gram-negative bacteria and destabilize the lipopolysaccharide (LPS) increasing the permeability of the bacterial membrane, leading to outflow of the cytoplasmic content and eventually cell death (Li *et al.*, 2006; Falagas and Kasiakou, 2005). They may also have a direct destabilizing effect in the inner membrane (Li *et al.*, 2005) and also inhibit vital, membrane bound respiratory enzymes (inhibition of type II NADH-quinone oxidoreductases [NDH-2]) (Deris *et al.*, 2013).

1.4.7.1 Resistance to polymyxins

The most common way of acquiring resistance to polymyxins is the alteration of the LPS via cationic substitution that prevents the binding of the cationic drug (Poirel *et al.*, 2017; Velkov *et al.*, 2010). This can be achieved by mutations in several chromosomal genes that are part of the complex system responsible for the synthesis of LPS (Fern´andez *et al.*, 2013; Loutet *et al.*, 2011). The most common polymyxin resistance mechanism in bacteria is due to the shielding of phosphates on lipid A with positively charged groups, such as phosphoethanolamine (pEtN) and L-4-aminoarabinose (L-Ara4N) (Fern´andez *et al.*, 2012; Breazeale *et al.*, 2005). The

complex pathways are regulated at multiple levels including the PhoP-PhoQ regulatory system encoded by phoP locus (Figure 10).

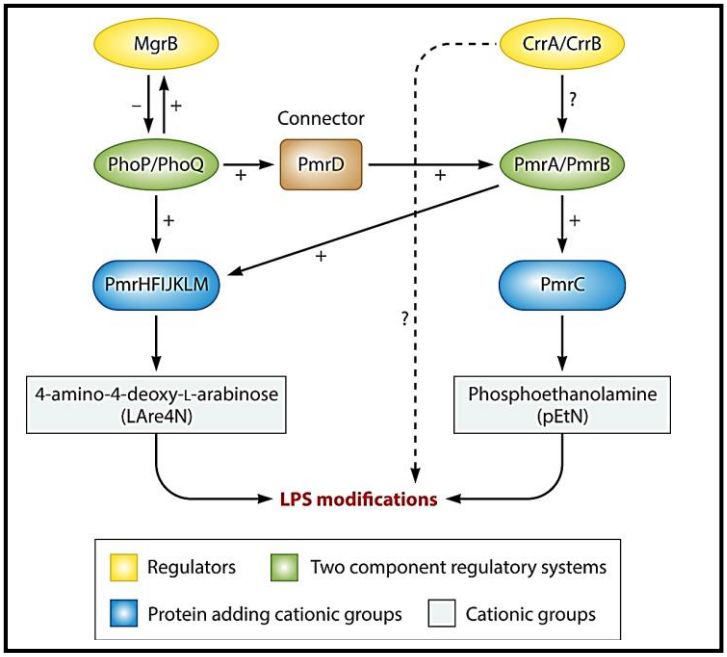

Figure 10: Regulation pathways of LPS modifications in Klebsiella pneumoniae (Poirel et al., 2017)

Recently, a plasmid-mediated colistin resistance gene, *mcr* (mobile colistin resistance) was discovered first for *E. coli* and *K. pneumoniae* isolates recovered in China between 2011 and 2014 (Liu *et al.*, 2016). The overall, production of MCR-1 in *E. coli* leads to an increase of the MICs of polymyxins. As a result, without any additional resistance mechanisms, the expression of MCR-1 is enough to confer resistance to colistin in *E. coli* and other enterobacterial species (Poirel *et al.*, 2017). Till date, several variants of this gene (*mcr*-1 till *mcr*-8) have been identified.

1.5 Aims and objectives

Long Term Aims

- To obtain the very first insight into the distribution of various types of *Klebsiella pneumoniae* causing blood-stream infections in the UAE

Specific Objectives

- To establish the antimicrobial susceptibility of *K. pneumoniae* causing bloodstream infections
- To assess the rate of and characterize Carbapenem Resistant *K. pneumoniae* (CRKP)
- To study the rate of hypervirulent strains among bloodstream causing *K. pneumoniae* (hvKP)
- To characterise these strains regarding their clonal types and virulence markers

To study the overlap between CRKP and hvKP isolates

Chapter 2: Material and Methods

2.1 Bacterial strains

A total of 125 non-repeat *K. pneumoniae* bloodstream isolates were collected in a two-year period, from January 2014 to December 2015, in Tawam Hospital (Microbiology Department), a tertiary care hospital in Al Ain City, United Arab Emirates (UAE). Duplicate isolates from the same patient were excluded and only the first isolate from each case was included. Initially, the strains were speciated using the standard microbiological procedure, i.e. VITEK® 2 ID-GN **(**BioMérieux, France**).** Isolates were stored in duplicates in Tryptic Soy Broth (TSB, Mast) containing 10% glycerol (Univar) at -80°C freezers. Prior each experiment strains were revived on Tryptic Soy Agar (TSA, Mast) and incubated for 24 h at 37°C.

2.2 Antibiotic susceptibility testing

For each pure isolate, the antimicrobial sensitivity testing was performed by disc diffusion technique as described in the guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2015). All stored *K. pneumoniae* isolates were freshly cultured on Tryptic-soy agar (TSA) plates. From the cultures, 0.5 McFarland density suspension was made in in sterile 1xPBS and this was spread onto ready-made Mueller-Hinton agar (MHA) plates (Pangulf Lab Solution) using a sterile cotton applicator swab (Citoswab, China) to provide semi-confluent growth. Antibiotic disks (MAST) were applied using antibiotic dispenser (MAST), seven discs per a 90 mm dish. All plates were incubated at 37°C for 18 h. Diameters of the zone of inhibition were measured with a caliper. The following antibiotics were used: Amikacin (AK), Ampicillin (AP), Amoxicillin-clavulanic acid (AUG), Aztreonam (AZT), Ceftazidime (CAZ), Chloramphenicol (CHL), Ciprofloxacin (CIP), Cefpodoxime (CPD), Cefotaxime (CTX), Doxycycline (DXT), Ertapenem (ERT), Fosfomycin (FOS),

Cefoxitin (FOX), Gentamicin (GN), Imipenem (IMP), Meropenem (MEM), Nalidixic acid (NA), Piperacillin-tazobactam (PTZ), Trimethoprim-sulphamethoxazole (SXT), Tetracycline (T) and Tobramycin (TOB).

For quantitative assay the broth microdilution assay was used. The quantitative susceptibility test was established against colistin (COL) only. Colistin was serially diluted in 100 µl volume of Muller Hinton Broth (OXOID) in 96 well microplates (Sarstedt, Germany) and inoculated by a Multipoint inoculator (MAST) to give a final concentration of 5 x 10^5 colony forming units (CFU)/ml. After 18 hours of incubation at 37°C the growth was visually assessed and the Minimum Inhibitory Concentration of the drug (MIC) was determined as the lowest concentration preventing visible growth of the organism tested.

Escherichia coli ATCC 25922 was used as control strain in all susceptibility testing, and *E. coli* ABC149, an mcr-1 positive isolate with colistin MIC of 4mg/L was additionally used in the colistin susceptibility testing.

Strains were considered to be multi-drug resistant if resistant to three or more classes of drugs (Magiorakos et al., 2012).

2.3 Strategy for screening for, and characterization of hypervirulent *Klebsiella pneumoniae*

All strains were screened by PCR for the presence of the *iucA* (Russo *et al.*, 2014) and *rmpA* (Brisse *et al.*, 2009) genes. Those positive for *iucA* were also tested for *rmpA2* (Yu *et al.*, 2015). A strain was considered to be hypervirulent if positive for *iucA* and for *rmpA* and/or *rmpA2*. The capsule type (Brisse *et al.* 2009, Turton *et al.*, 2010) and the MLST (Diancourt *et al.*, 2005) of the hvKP strains was also determined. hvKP and cKP strains were compared in terms of the frequency of the presence of

various siderophore genes, namely *kfuBC* (Brisse *et al.*, 2009), enterobactin (*entB*), salmochelin (*iroB*), Yersiniabactin (*irp2*) and aerobacting (*iucA*) (Russo *et al.,* 2014) and their positivity in string test (Patel *et al.*, 2014; Martin and Bachman, 2018).

2.4 Extraction of bacterial genomic DNA for PCR

Three to five colonies of isolates grown overnight on TSA plates were picked by toothpick and suspended into two 200 µl aliquots of sterile distilled water in Eppendorf tubes. The tubes were incubated for 10 minutes at 99°C on a thermo-block (Eppendorf). They were centrifuged for 10 minutes at 14,800 rpm; the supernatant was collected without touching the pellet, combined in a new autoclaved Eppendorf tube and kept at 4°C. This material was used for most experiments as the DNA target. For selected isolates, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturers' instruction.

2.5 Polymerase Chain Reaction (PCR)

All PCR reactions were performed on Applied Biosystems 2700 and 2720 thermocyclers. In all reactions *E. coli* J53RAZ genomic DNA and ultra-pure distilled water were used as negative controls. All positive reactions first obtained were confirmed by direct sequencing. The genes targeted, the primers and parameters used in different PCR reactions with respective references are listed in Table 9**.** PCR reactions were performed for 30-40 cycles at various annealing temperatures depending upon the melting temperature of the primer set used. Amplicons were analyzed on 1-2% agarose gels in the presence of ethidium bromide. Gels were photographed and scanned using the Biometra gel documentation system (Biometra, Gottingen, Germany). To assess the molecular mass of the amplicons Gene Ruler 100 bp DNA ladder (Fermentas) standards were run on each gel.

Table 9: Primers used in the study

Gene Targeted	Primers	Sequences (5'- 3')	PCR Product Size	Initial Denaturation	Cycle	Final Extension	References
Detection of selected beta-lactamase genes							
bla$_{CTX-M}$	MA-1 MA-2	SCS ATG TGC AGY ACC AGT AA CCG CRA TAT GRT TGG TGG TG	543bp	5' at 94°C	30X (30" at 94°C, 30" at 55°C and 60" at 72°C)	10'at 72°C	(Cao *et al.*, 2002)
bla$_{OXA-48-like}$	OXA-F OXA-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438bp	5' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	(Poirel *et al.*, 2011)
bla$_{KPC}$	KPC-F KPC-R	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798bp	5' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	(Poirel *et al.*, 2011)
bla$_{VIM}$	VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390bp	5'at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	(Poirel *et al.*, 2011)
bla$_{IMP}$	IMP-F IMP-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232bp	5' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	(Poirel *et al.*, 2011)
bla$_{NDM}$	NDM1-Fo NDM1-Re	TGCCGAGCGACTTGGCCTTG ACCGATGACCAGACCGCCCA	379bp	5'at 94°C	30X (30"at 94°C, 30" at 60°C, 60" at 72°C)	7' at 72°C	(Ghazawi *et al.*, 2012)

Table 9: Primers used in the study (continued)

Gene Targeted	Primers	Sequences (5'- 3')	PCR Product Size	Initial Denaturation	Cycle	Final Extension	References
Detection of aminoglycoside methyltransferase genes							
armA	armA-f armA-r	TATGGGGGTCTTACTATTCTGCCTAT TCTTCCATTCCCTTCTCCTTT	514 bp	5'at 94°C	40 X (15" at 94°C, 30" at 58°C, and 1' at 72°C)	10'at 72°C	(Fritsche *et al.*, 2008)
rmtA	rmtA-f rmtA-r	CTAGCGTCCATCCTTTCCTC TTTGCTTCCATGCCCTTGCC	635 bp				
rmtB	rmtB-f rmtB-r	TCAACGATGCCCTCACCTC GCAGGGCAAAGGTAAAATCC	459 bp				
rmtC	rmtC-f rmtC-r	GCCAAAGTACTCACAAGTGG CTCAGATCTGACCCAACAAG	752 bp				
rmtD	rmtD-f rmtD-r	CTGTTTGAAGCCAGCGGAACGC GCGCCTCCATCCATTCGGAATAG	376 bp				
rmtE	rmtE-f rmtE-r	ATGAATATTGATGAAATGGTTGC TGATTGATTTCCTCCGTTTTTG	818 bp	5'at 94°C	35 X (30" at 94°C, 60" at 55°C, and 1' at 72°C)	10'at 72°C	(Davis *et al.*, 2010)
rmtF	rmtF-f rmtF-r	GCGATACAGAAAACCGAAGG ACCAGTCGGCATAGTGCTTT	589 bp				(Hidalgo *et al.*, 2013)

Table 9: Primers used in the study (continued)

Gene Targeted	Primers	Sequences (5'- 3')	PCR Product Size	Initial Denaturation	Cycle	Final Extension	References
***Klebsiella pneumoniae* MLST**							
rpoB	VIC3 VIC2	GGC GAA ATG GCW GAG AAC CA GAG TCT TCG AAG TTG TAA CC	501 bp	5'at 94°C	35X (60" at 94°C, 60" at 50°C and 60" at 72°C)	10'at 72°C	(Diancourt *et al.*, 2005)
gapA	gapA173 gapA181	TGA AAT ATG ACT CCA CTC ACG G CTT CAG AAG CGG CTT TGA TGG CTT	450 bp	5'at 94°C	35X (60" at 94°C, 60" at 60°C and 60" at 72°C)	10'at 72°C	
mdh	mdh130 mdh867	CCC AAC TCG CTT CAG GTT CAG CCG TTT TTC CCC AGC AGC AG	477 bp	5'at 94°C	35X (60" at 94°C, 60" at 50°C and 60" at 72°C)	10'at 72°C	
pgi	pgi1F pgi1R	GAG AAA AAC CTG CCT GTA CTG CTG GC CGC GCC ACG CTT TAT AGC GGT TAA T	432 bp				
phoE	phoE604.1 phoE604.2	ACC TAC CGC AAC ACC GAC TTC TTC GG TGA TCA GAA CTG GTA GGT GAT	420 bp				
infB	infB1F infB1R	CTC GCT GCT GGA CTA TAT TCG CGC TTT CAG CTC AAG AAC TTC	318 bp				
tonB	tonB1F tonB2R	CTT TAT ACC TCG GTA CAT CAG GTT ATT CGC CGG CTG RGC RGA GAG	414 bp	5'at 94°C	35X (60" at 94°C, 60" at 45°C and 60" at 72°C)	10'at 72°C	(Diancourt *et al.*, 2005)

Table 9: Primers used in the study (continued)

Gene Targeted	Primers	Sequences (5'- 3')	PCR Product Size	Initial Denaturation	Cycle	Final Extension	References
Detection of *Klebsiella pneumoniae* virulence factor genes							
magA	mag A-F mag A-R	GGT GCT CTT TAC ATC ATT GC GCA ATG GCC ATT TGC GTT AG	1280 bp	5'at 94°C	35X (30" at 94°C, 60" at 50°C and 60" at 72°C)	7'at 72°C	(Lewis *et al.*, 2009)
rmpA	rmpA-F rmpA-R	ACT GGG CTA CCT CTG CTT CA CTT GCA TGA GCC ATC TTT CA	535 bp				
kfuBC	kfuB-F1179 kfuC-R649	GAA GTG ACG CTG TTT CTG GC TTT CGT GTG GCC AGT GAC TC	797 bp				
Capsule type K2	K2wzy-F1 K2wzy-R1	GACCCGATATTCATACTTGACAGAG CCTGAAGTAAAATCGTAAATAGATGGC	641 bp	5'at 94°C	35X (30" at 94°C, 90" at 58°C and 90" at 72°C)	10'at 72°C	(Turton *et al.*, 2010)
Capsule type K5	K5wzxF360 K5wzxR639	TGGTAGTGATGCTCGCGA CCTGAACCCACCCCAATC	280 bp				
Capsule type K54	wzxK54F wzxK54R	CATTAGCTCAGTGGTTGGCT GCTTGACAAACACCATAGCAG	881 bp				
Capsule type K57	wzyK57F wzyK57R	CTCAGGGCTAGAAGTGTCAT CACTAACCCAGAAAGTCGAG	1037 bp	5'at 94°C	35X (30" at 94°C, 90" at 58°C and 90" at 72°C)	10'at 72°C	(Turton *et al.*, 2010)
Capsule type K20	wzyK20F wzyK20R	CGGTGCTACAGTGCATCATT GTTATACGATGCTCAGTCGC	741 bp				

Table 9: Primers used in the study (continued)

Gene Targeted	Primers	Sequences (5'- 3')	PCR Product Size	Initial Denaturation	Cycle	Final Extension	References
Enterobactin *(entB)*	entB plus entB minus	GATGAAGACGATACCGTGC ACCGAATCCAGACCGTAGTC	388bp	5'at 94°C	35X (30" at 94°C, 30" at 56°C and 60" at 72°C)	7'at 72°C	(Russo *et al.*, 2014)
Salmochelin *(iroB)*	iroB plus iroB minus	ATCTCATCATCTACCCTCCGCTC GGTTCGCCGTCGTTTTCAA	234 bp				
Yersiniabactin *(irp2)*	irp2 plus irp2 minus	GCTACAATGGGACAGCAACGAC GCAGAGCGATACGGAAAATGC	229bp				
Aerobactin (*iucA*)	iucA plus iucA minus	AATCAATGGCTATTCCCGCTG CGCTTCACTTCTTTCACTGACAGG	340 bp	5'at 94°C	35X (30" at 94°C, 30" at 58°C and 120" at 72°C)	10'at 72°C	
rmpA2	rmpA2-F rmpA2-R	TGTGCAATAAGGATGTTACATTAGT TTTGATGTGCACCATTTTTCA	609 bp	5'at 94°C	35X (30" at 94°C, 30" at 55°C and 60" at 72°C)	10'at 72°C	(Yu *et al.*, 2015)

2.6 String test

The string test was conducted to check the hypermucoviscosity of *Klebsiella pneumoniae* colonies. The outcome result was defined as positive when an inoculation loop was able to generate a viscous string test of ≥5 mm in length from a colony on a blood agar plate after overnight incubation (Patel *et al.*, 2014; Martin and Bachman, 2018), meanwhile, a negative string test was assigned when the string was less than 5 mm in length or no string was formed at all.

2.7 Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to Gautom. Briefly, bacterial strains grown on TSA plates were suspended in 2ml of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH8.0) up to a density of 3 McFarland unit and suspensions were kept on ice. Simultaneously, 1% plug (low melting point) agarose (Sigma) was melted in 1% SDS in TE buffer (10mM Tris:1mM EDTA pH8.0) and kept at 54°C. 500 µl bacterial suspensions, 25 µl of proteinase K (Invitrogen) (20 mg/ml) and 525 µl of 1% plug agarose were combined, mixed carefully, quickly transferred into 1 ml syringes and kept for 15-30 minutes at room temperature to allow the solidification of the agarose. Aliquots of 5 ml cell lysis buffer (50 mM Tris: 50 mM EDTA pH 8.0, 1% Sarkosyl) and 25 µl proteinase K 20 mg/mL were distributed into 50 ml tubes and 1mm thick slices of agarose plugs were directly cut into them. They were incubated for 2 hours at 50°C in a shaking water bath (200 rpm). Subsequently, the plugs were washed twice with 10 ml of preheated sterile MilliQ water for 20 minutes in a 50°C shaker water bath. Plugs were washed four times each for 20 minutes with 10 ml of preheated TE buffer. Finally, plugs were stored in 5 ml of fresh TE buffer at 4°C.

Genomic DNA within the plugs were digested overnight at 37°C in a 100 µl restriction mixture made of 10 µl of NE buffer 4 (New England Biolabs), 1 µl of BSA (New England Biolabs), 30 U (1.5 µl) of *XbaI* enzyme (New England Biolabs) and 87.5 µl of sterile distilled water. Following digestion, the restriction mixtures were removed, and the plugs were incubated in 250 µl of 0.5X TBE buffer for 30 minutes at room temperature. Subsequently, plugs were inserted into wells of 1.4% of agarose gel (Pulse Field Running Agarose A2929, Sigma) prepared in 0.5xTBE buffer each gel contained *Salmonella* Braenderup strain H9812 as references standard for PFGE loaded on three wells: two sides and a middle one. Gels were run in CHEF Mapper (Biorad) electrophoresis chamber in 0.5X TBE buffer pre-chilled to 14°C. The running program consisted of 26 hours run at 6 V/cm with 120 angle and an initial switch time of 2.2 seconds and a final switch time of 54.2 seconds with linear ramp.

Gels were stained with ethidium bromide for 20 minutes, followed by de-staining in MilliQ water. Bands were detected and photographed under UV light in a Biometra gel documentation system. Gel pictures were stored as .tif files for further analysis. The GelCompare II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the banding patterns. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree graphically showing the level of relatedness between the isolates was created based on the Dice similarity coefficient (SD) (with a 1.5% position tolerance). For a routine analysis, strains showing patterns with SD $\geq$ 80% were arbitrarily considered to represent a pulsotype.

2.8 Multi-locus Sequence Typing (MLST)

In order to study the relatedness of *K. pneumoniae* strains, their genomic DNA were typed by MLST method based on the protocol described by (Diancourt et al., 2005) The seven housekeeping genes of seven *K. pneumoniae* (*ropB, gapA, mdh, pgi, phoE, infB, tonB*) were amplified by the primers shown in Table 9 *K. pneumoniae* isolates were assigned to sequence types using the tools onto the following webpage (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). All hvKP strains were subjected to MLST. For carbapenem resistant *K. pneumoniae* strains, if isolates formed a PFGE cluster with >4 members, selected members of the cluster were investigated by MLST. For this purpose, PFGE subtypes were assigned to each isolate exhibiting >90% PFGE pattern similarity, and at least one isolate from each subtype was MLSTed.

2.9 Sequencing

PCR products were purified by PCR purification kit (Promega) prior to sequencing. Sequencing was done using the Big Dye Cycle Terminator V.3.1 (Applied Biosystems) according to the manufacturer's instructions. The samples were sequenced in both directions using the 3130X Genetic Analyzer (Applied Biosystems). Sequences were analyzed using the MEGA5 (http://www.megasoftware.net/mega5/mega.html) and the Clone Manager v9 (Sci-Ed software) software.

2.10 Plasmid electrophoresis and localization of the *rmpA* gene by hybridization

Plasmids were isolated of the *rmpA* positive isolates by the alkaline lysis method of Kado and Liu (1981). Briefly, bacterial cells were collected from overnight culture on TSA plates, and suspended in 250µl of lysing solution (3%SDS, 50 µM Tris, pH 12.56-12.58). The suspensions were incubated in a thermoblock at 60°C for

45 minutes, with gentle mixing every 15 minutes, followed by addition of 250 µl of phenol-chloroform (1:1) and centrifugation at 13000 rpm for 15 minutes. About 100 µl of the top aqueous layer was transferred to another tube containing 5 µl loading dye. Thirty µl of these samples were loaded in 0.8% agarose gel and subjected to electrophoresis at 130 V for 4 hours. Gels were stained with ethidium bromide for 20 minutes followed by, de-staining with distilled water for 10 minutes. Rulers were placed around the gel, then photographed to help locate the bands using a gel documentation system (Biometra, Germany). For plasmid size control, *E. coli* 39R861 (Threlfall, Rowe, Ferguson, & Ward, 1986) were used. The gel was then depurinated in 0.25 M HCl, then denatured in 0.5 M NaOH, 1 M NaCl, and lastly neutralized in 1 M Tris, 0.6 M NaCl. All steps were repeated twice for 15 minutes, with gentle agitation, at room temperature; with the gels being rinsed with distilled water between the steps.

The gel was transferred to Hybond N+ nylon membrane using capillary action; with overnight soaking in 20XSSC (saline-sodium citrate). The following day, membranes were cross-linked using UV at 70 000 microjoules. Hybridization was done using the DIG DNA detection kit (Roche, Germany). *rmpA* probe previously labelled with the same kit was used. The nylon membrane was incubated in hybridization buffer, at hybridization temperatures for 30 minutes as a prehybridization step, after which the hybridization buffer containing the probe was added, incubated overnight at the hybridization temperature. Hybridization temperature was calculated based on the size and the GC ratio of the probe using the formula given by the manufacturer. On the next day, membranes were washed twice with 2XSSC/1%SDS for 5 minutes at room temperature, followed by washing twice in 0.1XSSC/1%SDS for 15 minutes at 68°C. Membranes were then rinsed in washing

buffer at room temperature incubated in blocking solution for 30 minutes, followed by incubation in antibody solution for 30 minutes. After that, membranes were washed twice in washing buffer, and equilibrated in detection buffer. Finally, membranes were incubated in color substrate solution, kept in the dark without shaking till the appearance of the labelled band.

2.11 Collection of clinical data

Data on the age, gender, nationality, underlying illness, prior antibiotic exposure of the patients were provided by Dr. Rayhan Hashmey (Tawam Hospital).

2.12 Ethical approval

The study was approved by the Al Ain Medical District Human Research Ethics Committee (AAMD/HREC) No. 14/03 – CRD 309/14.

2.13 Statistical analysis

Categorical variables were compared by two-tailed Fisher's test while the unpaired, two-tailed Student's *t* test was used to compare means. P value of <0.05 was considered statistically significant.

Chapter 3: Results

3.1 General characteristics of the group of patients

In total, 125 *K. pneumoniae* strains, the first such isolates were recovered from blood samples of individual patients and were included in the study. Information on gender, age, nationality and limited clinical data were available only for 122 patients.

The average age all patients included in the study was 45.58 ± 30.64 years and representing ages 61 male and 61 female patients with respective average ages of 47.66 ± 32.18 and 43.49 ± 29.13 years. The distribution of the strains by the patients' nationality is shown wards, by nationality of the patients are show in Table 10.

Table 10: Distribution of isolates by the patients' nationalities

Nationality	%	Nationality	%
Afghani	1.6	Palestinian	4.8
Bangladeshi	0.8	Saudi	0.8
Comoros Islandic	0.8	Serbian	0.8
Djiboutian	0.8	Sudanese	6.4
Egyptian	2.4	Syrian	2.4
Emirati	55.2	Yemeni	2.4
Ethiopian	0.8	No data	2.4
Philippine	1.6	Jordanian	2.4
Indian	3.2	Omani	1.6
Iraqi	2.4	Pakistani	5.6

As co-morbidities can considerably impact the type of infection acquired, the frequencies of selected co-morbidities, specifically diabetes mellitus, haematological malignancies and solid tumours among patients are shown in Table 11.

Table 11: The frequency of selected co-morbidities among patients

Co-morbidities	%
Diabetes mellitus	27.2
Malignancy	41.6
Haematological	10.4
Solid tumour	31.2
Diabetes and malignancy	14.4
None of the above	43.2
No data	2.4

Previous exposure to antibiotics may select organisms with high resistance profiles. Therefore, data were also collected on exposure to antibiotics within the previous one months. The data are summarised in Table 12.

Table 12: Previous exposure to selected groups of antibiotics

Antibiotic exposure	%
Any antibiotics	52.8
None	44.8
Any non-beta-lactams, only	2.4
Any beta-lactams	50.4
3rd gen. cephalosporins	5.6
Carbapenems	14.4
No data	2.4

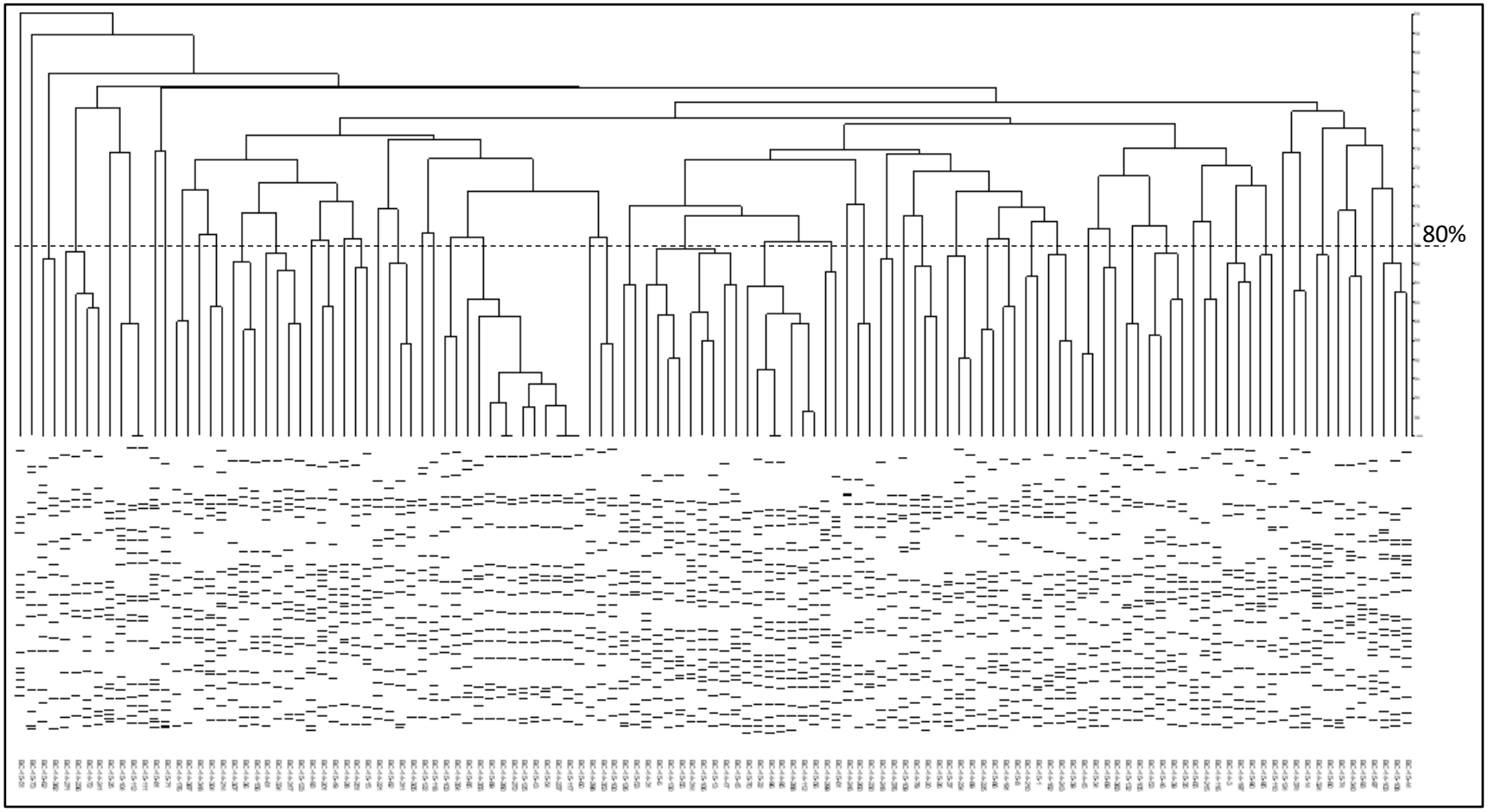

Figure 11: Macro-restriction analysis of the strains with pulsed field gel electrophoresis

3.2 Heterogeneity of the strains

First, the level of heterogeneity of the *K. pneumoniae* strains was established by macro-restriction analysis via PFGE (Figure 11).

The population was rather heterogenous forming 36 distinct clusters and 18 singletons. The size of clusters is shown in Table 13.

Table 13 : Clustering of the strains by macro-restriction analysis

Size of the clusters	Number of clusters
Singletons	18
Duplets	22
Triplets	8
Quadruplets	3
7 members	1
9 members	1
11 members	1

The analysis done if larger groups of strains also clustered in time and location. The time and space distribution of members of the larger clusters (≥ 4) is shown in Table 14.

Table 14: Distribution of larger clusters in time and space

PFGE cluster	Date of isolation	Ward
PF12	31-01-2014	Surgical Unit 2
	10-02-2014	Pediatric Intensive Care
	11-03-2015	Medical Unit 3
	27-04-2015	Pediatric Intensive Care
PF42	21-02-2014	Intensive Care Unit
	22-10-2014	Special Medical Unit
	01-11-2014	Intensive Care Unit
	29-11-2015	Oncology Unit 1
PF51	05-03-2014	Hematology Unit 2
	19-08-2014	Intensive Care Unit
	21-08-2014	Intensive Care Unit
	31-08-2015	Intensive Care Unit
PF27	21-03-2014	Pediatric Intensive Care
	21-03-2014	Intensive Care Unit
	08-04-2014	Intensive Care Unit
	29-09-2014	Emergency Department
	13-02-2015	Intensive Care Unit
	04-07-2015	Medical Oncology
	26-05-2015	Surgical Unit 1
PF28	24-01-2014	Surgical Unit 1
	02-02-2014	High Dependency Unit
	24-04-2014	Intensive Care Unit
	22-10-2014	Pediatric Intensive Care

Table 14: Distribution of larger clusters in time and space (continued)

PFGE cluster	Date of isolation	Ward
	10-01-2015	Hematology Unit 2
	22-01-2015	Intensive Care Unit
	22-04-2015	Intensive Care Unit
	26-05-2015	Surgical Unit 1
	22-10-2015	Pediatric Intensive Care
PF32	18-08-2014*	Intensive Care Unit
	04-09-2014*	Intensive Care Unit
	16-09-2014*	Intensive Care Unit
	18-11-2014*	Intensive Care Unit
	29-03-2015*	Oncology Unit 1
	21-05-2015*	Medical Unit 3
	08-06-2015*	Oncology Unit 1
	15-08-2015	Special Medical Unit 3
	02-09-2015*	Intensive Care Unit
	16-11-2015*	Special Medical Unit 3
	10-12-2015*	Intensive Care Unit

* *Strains marked are iucA positive, but were rmpA/A2 negative isolates, see later*

While limited clustering in time and space (e.g. PF32 in the Intensive Care Unit within a few weeks-times in 2014) did occur, no single locus explosive outbreak was indicated by the analysis.

This observation was further supported by performing MLST of selected members of PFGE clusters with >4 member. The sequence type (ST) of isolates

exhibiting <90% PFGE pattern similarity is shown in Table 15. MLST also confirmed the heterogeneity of isolates, as members of the PF27 cluster belonged to three STs, PF28 cluster had isolates of seven various STs, and even the most uniform PF32 group did not only have ST231 isolates but one isolates having ST34.

Table 15: Subtypes and multi-locus sequence types of isolates belonging to PF clusters with >4 members

UAEU No.	PFGE type based on >80% similarity	PFGE subtype based on >90% similarity	MLST	*gapA*	*infB*	*Mdh*	*pgi*	*phoE*	*rpoB*	*tonB*
BC-15-56	PF27	PF27a	NT	NT	NT	NT	NT	NT	NT	NT
BC-14-112		PF27a	ST29	2	3	2	2	6	4	4
BC-14-288		PF27b	ST29	2	3	2	2	6	4	4
BC-15-22		PF27c	NT	NT	NT	NT	NT	NT	NT	NT
BC-14-96		PF27c	ST714	2	3	2	2	6	4	160
BC-14-95		PF27c	ST714	2	3	2	2	6	4	160
BC-15-70		PF27d	ST711	2	61	2	2	6	4	4
BC-15-45	PF28	PF28a	ST3550	2	1	1	224	9	4	12
BC-14-47		PF28b	ST1770	18	22	18	63	11	13	179
BC-15-13		PF28c	ST23	2	1	1	1	9	4	12
BC-15-106		PF28d	ST334	18	22	18	22	25	37	51
BC-14-314		PF28e	ST35	2	1	2	1	10	1	19
BC-14-130		PF28f	ST23	2	1	1	1	9	4	12
BC-15-55		PF28f	ST3549	2	1	1	1	9	191	12
BC-15-61		PF28g	ST2433	2	106	4	158	248	1	19
BC-14-31		PF28h	ST23	2	1	1	1	9	4	12
BC-14-280	PF32	PF32a	ST231	2	6	1	3	26	1	77
BC-15-54		PF32a	ST231	2	6	1	3	26	1	77
BC-15-43		PF32a	ST231	2	6	1	3	26	1	77
BC-14-272		PF32a	NT	NT	NT	NT	NT	NT	NT	NT
BC-15-117		PF32a	NT	NT	NT	NT	NT	NT	NT	NT
BC-15-60		PF32a	NT	NT	NT	NT	NT	NT	NT	NT
BC-14-237		PF32a	NT	NT	NT	NT	NT	NT	NT	NT
BC-15-89		PF32a	NT	NT	NT	NT	NT	NT	NT	NT
BC-15-125		PF32a	NT	NT	NT	NT	NT	NT	NT	NT
BC-14-355		PF32b	ST231	2	6	1	3	26	1	77
BC-15-85		PF32c	ST34	2	3	6	1	9	7	4

3.3 Antibiotic resistance of the isolates

The susceptibility rates to specific antibiotics are shown in Table 16.

Table 16: Susceptibility rates of the isolates

Antibiotics	% Susceptible
Amoxicillin-clavulanate	67.2
Cefoxitin	81.6
Piperacillin–tazobactam	62.4
Ceftazidime	69.6
Ceftriaxone	65.6
Cefotaxime	65.6
Aztreonam	72.0
Ertapenem	88.0
Imipenem	88.8
Meropenem	88.8
Amikacin	86.4
Gentamicin	77.6
Tobramycin	72.8
Ciprofloxacin	77.6
Trimethoprim–sulfamethoxazole	65.6
Doxycycline	77.6
Chloramphenicol	77.6
Fosfomycin	100.0
Colistin	96.8

Based on these figures, 47 isolates (37.6%) were multi-drug resistant, *i.e.* resistant to three or more classes of antibiotics (Magiorakos *et al*., 2012). Those patients who received antibiotic therapy prior to the isolation of *K. pneumoniae* from the blood sample were significantly more likely to harbour an MDR isolate (P=0.0081). Other variables were not associated with having an MDR *K. pneumoniae*

infection (Table 16).

Furthermore, 15 isolates (12.0%) qualified as carbapenem resistant *K. pneumoniae* (CRKP), *i.e.* exhibiting non-susceptibility to at least one (always including ertapenem) of the carbapenems tested. Patients from whom CRKP were isolated were not exposed to antibiotics or to beta-lactam antibiotics more often than other patients (P=0.0503 and 0.0986). However, they were highly significantly more likely to receive prior carbapenem therapy (P=0.0098). No correlation between being infected with a CRKP strain and the presence of diabetes mellitus or any malignancy or being Emirati or non-Emirati was found either (P=1.0, 0.4059, 0.7887, respectively) (Table 17).

Table 17: Correlation of different variables with having a multi-drug, or carbapenem resistant K. pneumoniae (CRKP) infection

Variables	**MDR (n=44)#**	**Non MDR (n=78)**	**P**
Prior antibiotic therapy	31	35	0.0081
Malignancy	21	31	0.4478
Diabetes mellitus	11	26	0.4138
Emirati nationality	23	46	0.5688
Variables	**CRKP (n=15)**	**CSKP (n=107)#**	**P**
Prior antibiotic therapy	12	54	0.0503
Prior beta-lactam therapy	11	52	0.0986
Prior carbapenem therapy	6	12	0.0098
Malignancy	8	43	0.4059
Diabetes mellitus	4	30	1.0
Emirati nationality	8	61	0.7887

 # n is the number of patients whose data were available
MDR: Multi-drug resistant, CRKP: Carbapenem resistant K. pneumoniae, CSKP: carbapenem susceptible K. pneumoniae

3.4 Susceptibility of the carbapenem resistant isolates to non-beta-lactam antibiotics

Although the most common mechanisms causing resistance to carbapenems in *Enterobacteriaceae* does not cause resistance to non-beta-lactams antibiotics, per se, often carbapenem resistant strains exhibit non-susceptibility to several drugs of different classes. The comparison is done for the susceptibilities of strains to such antibiotics. As can be seen on Table 18, with the exception of fosfomycin and colistin, CRKP strains were significantly less often susceptible than their carbapenem susceptible, CSKP, counterparts.

Table 18: Comparative susceptibilities of carbapenem resistant (CRKP) and carbapenem susceptible (CSKP) Klebsiella pneumoniae to non-beta-lactam antibiotics

Antibiotics	% Susceptible		Significance (P)
	CRKP	CSKP	
Amikacin	6.7	98.2	< 0.0001
Gentamicin	13.3	86.4	< 0.0001
Tobramycin	0.0	82.7	< 0.0001
Ciprofloxacin	6.7	87.3	< 0.0001
Trimethoprim–sulfamethoxazole	6.7	73.6	< 0.0001
Doxycycline	53.3	79.1	0.0482
Chloramphenicol	6.7	87.3	< 0.0001
Fosfomycin	100.0	100.0	1.0
Colistin	86.7	98.2	0.0703

3.5 Molecular characterization of carbapenem resistant *Klebsiella pneumoniae*

The mechanism of carbapenem resistance was determined by subjecting the isolates to PCR targeting the most common carbapenemase enzymes. The results are shown in Table 19. All strains produced at least one carbapenemase, with the exception of one that expressed both NDM and an OXA-48-like genes.

Table 19: Distribution of carbapenemase genes in CRKP isolates

Carbapenemase produced	%
$bla_{\text{OXA-232}}$	73.3
$bla_{\text{OXA-162}}$	6.7
$bla_{\text{NDM-1}}$	6.7
$bla_{\text{NDM-1}}$ + $bla_{\text{OXA-232}}$	6.7
bla_{VIM}	6.7

Eleven (73.3%) of the CRKP strains carried a $bla_{\text{CTX-M}}$ gene, while the same figure among the CSKP isolates was 20.1% (P=0.0001).

As with the exception of two CRKP strains, all isolates exhibited non-susceptibility to all the aminoglycosides tested. Exploring the presence of 16S methylase genes and with exception of the above mentioned two isolates, all CRKP strains carried such genes. Actually, 10 (69.1%) carried *rmtF,* while 3 (20%) carried *armA.* In none of the CSKP isolates could detect any 16S methylase genes, irrespective of the spectrum of their aminoglycoside resistance.

No mobile colistin resistance genes were detected in any of the colistin resistant strains.

The macrorestriction analysis of the CRKP strains revealed that beyond 5 singletons, 10 of them belonged to a single cluster, PF32. The MLST analysis revealed that two singletons belonged to ST14, further 3 belonged to ST23, ST307 and ST70, respectively. The 10 member-strong cluster was made up by ST231 isolates, all expressing the $bla_{\text{OXA-232}}$, *rmtF* genotype.

3.6 Screening for hypervirulent strains

All strains were screened for the presence of *rmpA* and *iucA* genes and those carrying *iucA* were also tested for *rmpA2*. Strains carrying *iucA* and *rmpA* and/or *rmpA2* were considered hypervirulent. Of the 125 strains tested, only 13 (10.4%) fulfilled the criteria.

3.7 Characterization of hypervirulent strains

3.7.1 Molecular typing of hypervirulent strains

In order to assess more subtle relationships between hvKP strains their PFGE patterns were reanalysed without the effect of the large number of cKP outliers. As seen in Figure 12, 6 strains formed a large cluster (previously assigned to PFGE patterns PF28 in Figure 11) while two strains (previously belonging to PF27) formed a pair. The remaining hvKP strains were singletons.

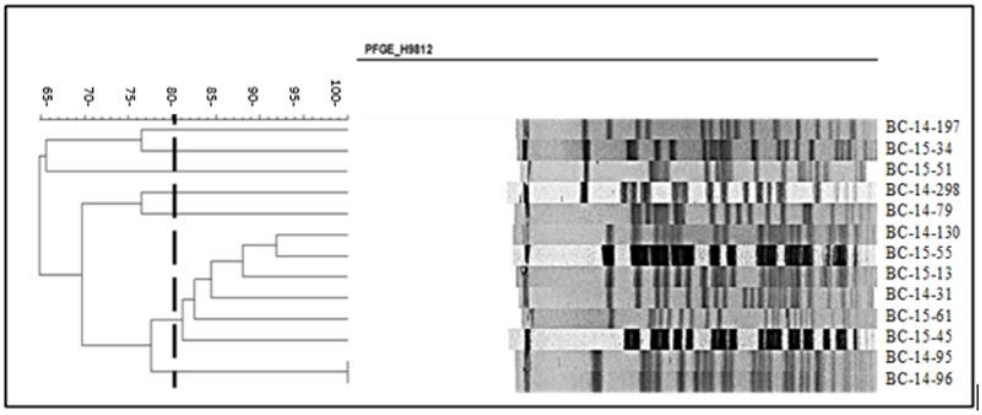

Figure 12: Macro-restriction patterns of the hvKP strains-dashed line indicates 80% similarity

MLST analysis of the strains revealed that the 6 isolates clustering together all belonged to ST23 or CC23, i.e. single locus variants (SLV) of ST23, while the duplets were ST714 (Table 20).

Table 20: Sequence types, date and location of isolation of hvKP strains

Strains	Sequence type	Date of isolation	Ward
BC-15-61	ST23	16-06-2015	Special Medical Unit 3
BC-14-130	ST23	24-04-2014	Intensive Care Unit
BC-14-31	ST23	24-01-2014	Surgical Unit 1
BC-15-13	ST23	22-01-2015	Intensive Care Unit
BC-15-55	ST3549-CC23	26-05-2015	Surgical Unit 1
BC-15-45	ST3550-CC23	22-04-2015	Intensive Care Unit
BC-14-197	ST36	05-07-2014	Intensive Care Unit
BC-15-34	ST380	09-03-2015	Emergency Department
BC-15-51	ST380	11-05-2015	Oncology Unit 1
BC-14-298	ST65	08-10-2014	VIP Unit
BC-14-95	ST714	21-03-2014	Intensive Care Unit
BC-14-96	ST714	21-03-2014	Pediatric Intensive Care Unit
BC-14-79	ST86	11-03-2014	Intensive Care Unit

As can be seen in Table 20, strains were not recovered from an explosive outbreak, but the distribution of isolation times and location are rather heterogenous.

3.7.2 The capsule type, string test positivity and the presence of siderophore genes

The capsule type, positivity in string test and the presence of various iron acquisition genes are shown in Table 21.

Table 21: Capsule type, string test and iron acquisition genes of hvKP strains

Strain	Sequence type	Capsule type	String test	Siderophore genes
BC-15-61	ST23	K1	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-14-130	ST23	K1	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-14-31	ST23	K1	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-15-13	ST23	K1	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-15-55	ST3549-CC23	K1	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-15-45	ST3550-CC23	K1	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-14-197	ST36	ND	neg	*iucA, entB, iroB, irp2*
BC-15-34	ST380	K2	pos	*iucA, kfuBC, entB, iroB, irp2*
BC-15-51	ST380	K2	neg	*iucA, kfuBC, entB, iroB, irp2*
BC-14-298	ST65	K2	pos	*iucA, entB, iroB, irp2*
BC-14-95	ST714	ND	neg	*iucA, entB, iroB, irp2*
BC-14-96	ST714	ND	neg	*iucA, entB, iroB, irp2*
BC-14-79	ST86	K2	neg	*iucA, entB, iroB, irp2*

The string test was uniformly positive in all K1 capsule type strains and yielded varying results with the K2 isolates, but was negative with the other strains. The most common siderophore systems were present in all isolates, and the majority of them carried the ABC transporter *kfuBC* genes, as well.

The comparative respective figures found in cKP strains are shown in Table 22. While hvKP isolates carried as many as 4.53 ± 0.66 iron acquisition related genes per strains, the same figure among cKP was 1.94 ± 0.92, only. With the exception of enterobactin gene *entB*, present in all strains, all other iron acquisition related genes were significantly more common among hvKP isolates. Nevertheless, the only gene clearly distinguishing between the two group of strains was *iroB*, part of the salmochelin siderophore system.

Table 22: Frequency of iron acquisition system genes and string test positivity among cKP and hvKP strains

Gene / Feature	**% positivity**		**Significance (P)**
	cKP	**hvKP**	
Genes			
iucA	9.8	100.0	<0.0001
kfuBC	30.4	61.5	0.0322
entB	100.0	100.0	1.0
iroB	0.0	100.0	<0.0001
irp2	54.5	100.0	0.0008
Feature			
String test	3.6	61.5	<0.0001

It was noteworthy that none of the String test positive cKP strains carried any iron acquisition related genes beyond the omni-present *entB*.

3.7.3 The location of the *rmpA* gene

Studying the location of the *rmpA* gene in the hvKP isolates. Hybridizing the plasmid electrophoresis gel of the strains with a *rmpA* specific probe revealed that in all isolates the gene was located on plasmids of varying size, but all were large (>150 kb) in size (Figure 13).

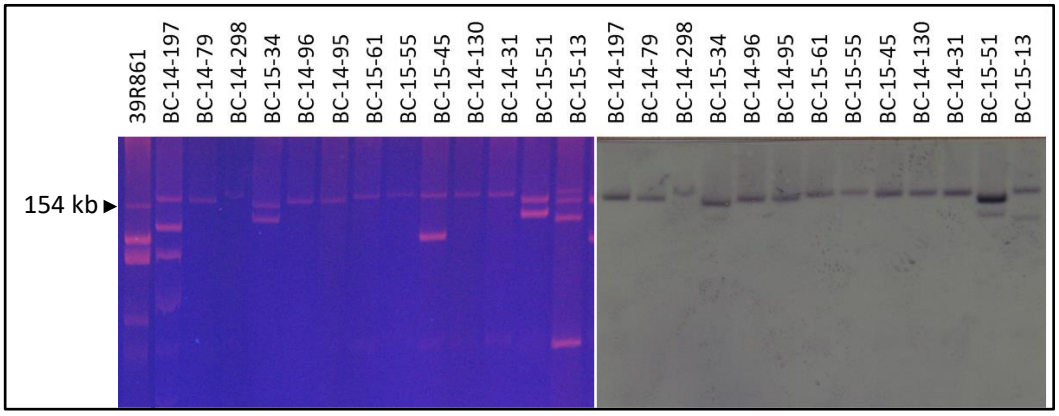

Figure 13: Localization of the rmpA gene 39R861: Plasmid molecular weight standard

3.7.4 Antimicrobial susceptibility of hypervirulent strains

Analysing the antimicrobial susceptibility of hvKP strains revealed that, in general, they were highly susceptible (Table 23). There was one single exception, a carbapenem resistant, i.e. CRKP, in which the highly virulent character was combined with an extensive resistance pattern. It was noteworthy, that despite its broad range of non-susceptibility (AMC, PTZ, FOX, CPD, CAZ, CTX, IMI, MEM, ERT, SXT, AK, TOB, DOX), this strain (BC-15-13) still exhibited susceptibility to colistin. On the other hand, one isolates (BC-15-34) while universally susceptible, was resistant to the polymyxin.

The CR hvKP strain carried a *bla*$_{VIM}$ carbapenemase gene, which represented a new allele, VIM-55 (GenBank MG552720).

Table 23: Rate of susceptibilities of hvKP strains

Antibiotics	% Susceptible
Amoxicillin-clavulanate	84.6
Cefoxitin	84.6
Piperacillin–tazobactam	61.5
Ceftazidime	84.6
Ceftriaxone	84.6
Cefotaxime	84.6
Aztreonam	92.3
Ertapenem	92.3
Imipenem	92.3
Meropenem	92.3
Amikacin	92.3
Gentamicin	100.0
Tobramycin	84.6
Ciprofloxacin	100.0
Trimethoprim–sulfamethoxazole	92.3
Doxycycline	92.3
Chloramphenicol	92.3
Fosfomycin	100.0
Colistin	92.3

3.8 Characteristics of patients carrying hypervirulent *Klebsiella pneumoniae*

Patients suffering from hvKP infections were significantly more likely to have diabetes and were significantly less likely to have received previous antibiotic therapy. Although the frequency of being Emirati was considerably more common among patients with cKP infections, the difference did not reach statistical significance (Table 24).

Table 24: Comparison of patients' characteristics with cKP and hvKP infections

Gene / Feature	% positivity		Significance (P)
	cKP*	hvKP	
Co-morbidities			
Diabetes	22.0	76.9	0.0001
Malignancy	43.1	38.5	1.0
Nationality			
Age	44.1 ± 33.45	55.6 ± 22.96	0.2116**
Emirati	58.7	38.5	0.2367
Antibiotic exposure			
Any antibiotics	57.8	23.1	0.0208
Any beta-lactams	55.0	23.1	0.0807
Carbepenems	14.7	15.4	1.0

* *109 patients for whom data were available*
** *Student's t test*

3.9 *iucA* positive but *rmpA/A2* negative strains

Eleven strains, while carrying the *iucA* gene, were *rmpA/A2* negative and hence, they did not qualify as hvKP. Nevertheless, they carried a broad panel of iron-acquisition genes. The average number of such genes per strain was 3.90 ± 0.30 compared to that seen in hvKP (4.53 ± 0.66), and much higher than seen in the 101 *iucA* negative cKP strains (1.73 ± 0.69). With the exception of one ST502 isolate, they

formed a single PFGE cluster (PF32) and represented ST231. These 10 strains were all carbapenem resistant carrying a *bla*$_{\text{OXA-232}}$ gene and the *rmtF* 16S methylase gene. All of them exhibited a broad range of resistance, two of them being non-susceptible to colistin, too.

The time and location distribution of their isolation is shown in Table 14 (the *iucA* positive, but *rmpA/A2* negative isolated are marked by a *).

Chapter 4: Discussion

During the two-year-long collection period 125 cases of *Klebsiella pneumoniae* bloodstream infection (BSI) were identified in our 461-bed tertiary care hospital. As most of the literature deals with MDR *K. pneumoniae* bloodstream infections, it is difficult to find general data on the incidence of *K. pneumoniae* BSI. Furthermore, even if data were available it is problematic to compare the incidence in variable clinical settings. Nevertheless, the rate of *K. pneumoniae* BSI during a two-year-long period was high in our setting, if compared it to a 1420-bed tertiary-care teaching hospital in Northern Italy, where 217 unique patient *K. pneumoniae* BSIs were described during a same time-period (Girometti *et al.*, 2014). This higher rate could possibly be explained by the fact that a large proportion of our patients suffered of either a solid tumour or of a haematological malignancy, and the BSI could have been the result of a mucosal barrier injury. It is especially plausible, as it has been shown that *Klebsiella pneumoniae* infections often originate from the patient's own intestinal microbiota (Gorrie *et al.*, 2017). Since our study was a retrospective investigation of *K. pneumoniae* isolated from bloodstream, unfortunately there is no data on the intestinal carriage of *K. pneumoniae* by these 125 patients to support this hypothesis.

Of the 125 *K. pneumoniae* isolates 47 (37.6%) qualified as MDR using the European Centre for Disease Prevention and Control criteria (Magiorakos *et al.*, 2012). This is a slightly higher figure than the one reported recently from South Korea (31.3%) (Kim *et al.*, 2019), but substantially lower than the 64% reported from the above mentioned healthcare facility in Northern Italy (Girometti *et al.*, 2014). The non-susceptibility to carbapenems in our collection, has not reached the Italian level of

47%, however, it is markedly higher than the South Korean CRKP rate (12% in our hospital vs. 3.1% in South Korea) (Girometti *et al.*, 2014; Kim *et al.*, 2019). It is noteworthy that prior antibiotic therapy was linked to the isolation of MDR *K. pneumoniae*. Prior carbapenem therapy, on the other hand, was significantly linked to the isolation of a CRKP. Our findings confirm similar observations reported earlier from multiple geographical regions (Liu *et al.*, 2018; Park *et al.*, 2012).

Despite the fact that *K. pneumoniae*, especially MDR *K. pneumoniae*, is prone to spread within the hospital settings (Hendrik *et al.*, 2015), in this study the PFGE and MLST analysis has revealed a large variety of *K. pneumoniae* strains causing BSI, with only three clusters having >4 members exhibiting >80% similar PFGE patterns (Figure 11). These results are suggestive of effective infection prevention and control program in our hospital. On the other hand, assuming that these *K. pneumoniae* strains likely originated from the patients' own gastrointestinal tract (Gorrie *et al.*, 2017), the findings also underline the importance of screening for colonisation of high risk patients.

Revealing colonisation is especially important in the lights of the relatively high proportion of carbapenem resistant *K. pneumoniae* causing bloodstream infections, with predominance of OXA-232 producing *K. pneumoniae* ST231 carrying aerobactin, yersiniabactin and enterobactin siderophore genes. Carrying three or four siderophores was associated with being more virulent in a large study comparing virulence of *K. pneumoniae* (Holt *et al.*, 2015), therefore the clonal spread of carbapenem resistant *K. pneumoniae* ST231, not only multidrug resistant but also virulent, is a serious concern. Although the *K. pneumoniae* ST231 clone is not considered an international high risk clone (Navon-Venezia *et al.*, 2017), it has been

reported to carry *bla*$_{\text{OXA-232}}$ and to disseminate rapidly causing outbreaks in Brunei (Abdul Momin *et al.*, 2017) and in India (Shankar *et al.*, 2019). Sporadic OXA-232 producing ST231 isolate was also reported from Switzerland (Mancini *et al.*, 2018). As only limited time and location overlap was observed within this cluster of strains, such isolates were either repeatedly introduced into our institution or they had a common source yet to be revealed, but investigation of either of these hypotheses was beyond the scope of the study.

The hypothesize is that this latter group of virulent and highly resistant isolates could also be considered as just being one step away to become really hypervirulent, i.e. by acquiring the plasmid-encoded *rmpA/A2* genes. Only further surveillance work encountering ST231 hvKP otherwise closely related to these isolates could prove our assumption about the potential dangers of the evolvement of this highly resistant, hypervirulent variety.

The other NDM and/or OXA-48-like producer isolates in the collection were diverse: OXA-232 or NDM-1 and OXA-232 co-producer ST14, OXA-162 producer ST307 and an NDM-1 producer ST70. The former two clonal lineages of *K. pneumoniae* are recognized as important international outbreaks clones (Navon-Venezia *et al.*, 2017), and both has been described already from the UAE to produce NDM-1 or OXA-48-like carbapenemases, or co-produce these carbapenemases. (Al-Baloushi *et al.*, 2018; Moubareck *et al.*, 2018; Mouftah *et al.*, 2019; Sonnevend *et al.*, 2015).

ST70 is not considered a high-risk epidemic clone, however it was reported to carry *bla*$_{\text{CTX-M-type}}$ ESBL gene in Bulgaria (Markovska *et al.*, 2017), *bla*$_{\text{KPC-2}}$ in Argentina and in Brazil (Jure *et al.*, 2019; Seki *et al.*, 2011) and recently also to harbour

bla$_{NDM-1}$ in Greece (Politi *et al.*, 2019). In all these reports *K. pneumoniae* ST70 occurred sporadically, but the presence of this clone in South America, the Balkan and now in the Gulf Region shows its capability to acquire genes coding for broad spectrum beta-lactam hydrolysing genes including various carbapenemases.

Isolates of the other larger PFGE cluster, PF27 (Table 15) belonged to ST29, ST711 and ST714, which are all part of the clonal complex CC29. Members of this clonal complex were described to be ESBL (Surgers *et al.*, 2019), or carbapenemase producers OXA-48 (Uz Zaman *et al.*, 2014), NDM-1 (Mukherjee *et al.*, 2019), and also being hypermucoviscous and hypervirulent (Turton *et al.*, 2019). This latter hypervirulent subtype of CC29 were reported to have K54 capsular serotype. In our collection two *K. pneumoniae* ST714 isolates of this clonal group possessed *rmpA/A2*, salmochelin, aerobactin and yersiniabactin, but PCR for the K54 serotype did not detect the relevant gene, and the isolates were not hypermucoviscous by string test. Despite of these facts, these two isolates were considered hypervirulent based on their genotypes as suggested by Russo and Marr (2019). The hypervirulent ST714-CC29 isolates remained susceptible to 3rd generation cephalosporins, but other members of the group with ST29 and ST711 were ESBL producers and carried the *bla*$_{CTX-M}$ gene. Consequently, although PFGE clustered these isolates, they were rather heterogenous. Even though, OXA-48 carbapenemase ST29 isolates were reported to cause outbreak in the region (Zaman *et al.*, 2014), in our collection all CC29 isolate remained susceptible to carbapenems.

The third large PFGE cluster, PF28 also contained several isolates with different sequence types (Table 15), further confirming the heterogeneity of our *K. pneumoniae* collection. It was noteworthy though that five members of this cluster of

nine isolates belonged to ST23 or its single locus variants ST3549 and ST3550. These two sequence types were novel, first reported by us. ST23, or rather the clonal complex CC23 is considered as the prototype of hypervirulent *K. pneumoniae*. (Wyres *et al.*, 2019) Indeed, these 5 isolates of PF28 cluster harboured the *rmpA/A2*, salmochelin, aerobactin and yersiniabactin genes, had K1 serotype and were phenotypically hypermucoviscous (Table 21). In addition to these CC23 isolates, identified a further string test positive, genotypically hypervirulent *K. pneumoniae* ST23 with capsular type K1, exhibiting a slightly different PFGE profile. Of the six CC23 isolate five were susceptible to all of the antibiotics tested, but one isolate BC-15-13 was resistant to 3rd generation cephalosporin, carbapenem, co-trimoxazole, amikacin, tobramycin, tetracycline and chloramphenicol. BC15-13 carried a novel VIM allele by PCR and sequencing: VIM-55. This allele differs from VIM-19 by one amino acid, at position 229 in VIM-55 an arginine replaces the histidine found in VIM-19. Exploring the genetic support and hydrolytic activity of this novel enzyme was beyond the scope of my research, further ongoing studies should reveal these features. Carbapenemase producer *K. pneumoniae* ST23 is a rare finding, although two members of the clone were reported to produce OXA-48 or NDM-1 from the United Kingdom (Turton et al., 2018) (Roulston et al., 2018). VIM-producing members of the clone were only recently encountered in China (*bla*$_{\text{VIM-1}}$) (Dong *et al.*, 2019) and in Iran (*bla*$_{\text{VIM-2}}$). (Mohammad *et al.*, 2018) Nevertheless, finding of an isolate, which is MDR and hypervirulent is a most feared scenario, as such bacteria are truly “superbugs”, with a great capacity to cause disease and at the same time being extremely difficult to treat.

Further to identifying hypervirulent *K. pneumoniae* of the CC23 and CC29, it has been described here hvKP belonging to sequence types ST36, ST380, ST65 and

ST86, all possessing *rmpA* on plasmid, four siderophore genes, and all but the ST36 isolate having capsular type K2 (Table 21).

K. pneumoniae ST36 has been reported to be present in several continents, is a known hvKP causing bacteremia, and it has also been reported to produce KPC-2 from China. (Feng *et al.*, 2018) Our ST36 isolate was non-susceptible to piperacillin-tazobactam, to 3rd generation cephalosporins and to monobactams, but remained susceptible to carbapenems and non-beta-lactam antibiotics.

The predominant serotype K2 hypervirulent clones *K. pneumoniae* clones are the ST65, ST86, and ST380 (Bialek-Davenet *et al.*, 2014), and members of all of these three clones were present and caused bloodstream infection in our institution. Isolates belonging to these clones were susceptible to antibiotics, i.e. they were typical hvKP.

Hypervirulent *K. pneumoniae* infection, commonly encountered in the Pacific Rim of Asia and in China (Paczosa and Mecsas, 2016), has only been sporadically reported from the Gulf region. A few cases of infections caused by hypermucoviscous *K. pneumoniae* were reported from Saudi Arabia (Enani and El-Khizzi, 2012) and Oman (Basu *et al*., 2009), without any molecular characterization. Therefore, to the best of our knowledge, our observation is the first in the region describing hvKP strains causing bloodstream infections. It is noteworthy, that like the majority of cKP, the hvKP isolates were also diverse.

In line with the earlier observations (Li *et al.*, 2018; Russo and Marr, 2019), patients with diabetes, and those who did not received antibiotic therapy prior to the bloodstream infection were significantly more likely to suffer of an hvKP infection in our setting as well. Unfortunately, although reports on hvKP infections emphasis the

ability of such strains to cause metastatic abscesses (Russo and Marr, 2019), no clinical information on metastatic infections, e.g. endophtalmitis, meningitis or liver abscess, neither on the 30-day-mortality were available to us.

Defining hvKP is still a controversial issue, and the approach followed in our study by relying only on the genomic definition is widely accepted (Li *et al.*, 2018). Further studies to complement our findings should confirm our, genomic definition of hypervirulence by performing *in vivo* studies. However, genetically hypervirulent isolates did not always exhibit full virulence in *in vivo* (Fu *et al.*, 2019) but surmised the virulence potential of the isolates' as all of them caused an invasive infection, i.e. were from the bloodstream.

Chapter 5: Conclusions

The majority of *K. pneumoniae* isolated from the two-year-long study period exhibited diverse molecular fingerprints. More than a third of the isolates were multi-drug resistant, and isolation of an MDR *K. pneumoniae* infection was more likely if the patient had previous exposure to antibiotics. High carbapenem resistance rate was detected with the predominance of OXA-232 producing *K. pneumoniae* ST231. Over 10% of the collection was hypervirulent *K. pneumoniae* based on genomic definition, i.e. the presence of plasmid-borne *rmpA*, salmochelin, aerobactin and yersiniabactin genes. The hypervirulent isolates were also diverse, belonging to ST23, ST3549, ST3550, ST36, ST380, ST65, ST714 and ST86. The presence of ST231 CRKP strains carrying the siderophore genes otherwise characteristic to hvKP, but lacking the *rmpA/A2* gene needs further attention as, at least theoretically, the mere acquisition of a plasmid carrying the *rmpA/A2* gene could convert these highly resistant strains to real hvKP. Diabetic patients and those who did not receive prior antibiotic therapy were more likely to have hypervirulent *K. pneumoniae* infection. The majority of hypervirulent *K. pneumoniae* were susceptible to antibiotics, however an ST23 isolate was carbapenem resistant due to the production of a novel metallo-beta-lactamase, VIM-55.

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